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Le Président de l'Office européen des brevets
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High-active glycoproteins - process conditions and an efficient method for their production

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High-active glycoproteins - Process Conditions and an Efficient Method for their production

The present invention relates to human active glycoproteins, a pharmaceutical composition for use in therapy comprising the glycoproteins, a method for optimized or differential sialylation of the glycoproteins, a method for the determination (i) of highly active glycoproteins and for the determination (ii) of production conditions of the glycoproteins, and the invention relates to the use of the glycoproteins for prophylactic and/or therapeutic treatment of diseases, particularly bone marrow transplantation, Neutropenia, Cytopenia, AML and myelodysplastic syndromes, cancer, HIV and/or diseases of hemotopoietic systems.

An important aspect of the present invention is therefore the platform technology for

- a process
    - for the production of different sialylation forms of glycoproteins, and

- for the production of forms of the glycoprotein with different activity including highest activities
- a process for the determination of production conditions of highly active glycoproteins, and
- a process for the determination of highly active glycoproteins
- a process for determination of the sialylation form(s) of the glycoprotein with optimized pharmacokinetic properties
- a process for the production of the glycoprotein with optimized pharmacokinetic properties

The immune system plays a critical role in the pathogenesis of a wide range of important diseases and conditions, including infection, autoimmunity, allograft rejection and neoplasia. The shortcomings of the immune system in these disorders can be broadly considered as either the failure to develop a sufficiently potent response to a deleterious target or the inappropriate generation of a destructive response against a desirable target. Standard medical treatments for these diseases, including chemotherapy, surgery and radiation therapy, have clear limitations with regard to both efficiency and toxicity. While prevention of the disease or condition would be ideal, these approaches typically have met with little success. A well known therapeutic manipulation of the immune response of a patient is the treatment with recombinant glycoproteins, particularly cytokines e.g. GM-CSF, IL-2, TNF-alpha, G-CSF, JE, IL-7 and antibodies. Unfortunately, the reduced

biological activity of the conventional recombinant glycoproteins is a problem in this strategy.

Therefore, the technical problem underlying the present invention is to provide (a) highly active glycoproteins, (b) a method for their production and (c) a method for the determination of highly active glycoproteins and (c') for the determination of conditions of production of the glycoproteins with improved serum-half-life, pharmacokinetics and immunogenicity as well as (d) the use of said glycoproteins in diagnostic or immunogenic compositions.

This problem is solved by the provision of the embodiments as defined in the claims, especially by a method for producing highly active human glycoproteins by differential sialylation.

It has been surprisingly discovered that a special sialylation degree of human proteins is responsible for an improved biological activity. Glycoproteins produced by the methods of the invention are more active and more effective biological products than native glycoproteins or recombinant carbohydrate mutants of said glycoproteins. The present invention therefore relates to human highly/higher active glycoproteins and a pharmaceutical composition for use in therapy comprising the glycoproteins.

In addition, the methods can be effectively used to generate glycoproteins with an optimized serum-half life, pharmacokinetics and immunogenicity.

Accordingly, the present invention provides a method for production of a glycoprotein having the ability to stimulate an immune response, in particular the growth and differentiation of primate hematopoietic progenitor cells

The subject matter of the invention are also methods for the determination (i) of highly active glycoproteins or for the determination (ii) of production conditions of said glycoproteins.

Also claimed is a kit for enhancing an immunogenic response of a mammal to antigens in a vaccine comprising the glycoprotein, and/or synthetic analogues, modifications and pharmacologically active fragments thereof and an information about the using of parts of the kit.

Before the present compositions, formulations and methods are described, it is to be understood that this invention is not limited to the particular methods, compositions, and cell lines described herein, as such methods, compositions, and cell lines may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which is only defined by the appended claims.

As used herein, including the appended claims, singular forms of words such as "a," "an," and "the" include their corresponding plural referents unless the context clearly dictates otherwise. Thus, e.g., reference to "an organism"

includes one or more different organisms, reference to "a cell" includes one or more of such cells, and reference to "a method" includes reference to equivalent steps and methods known to a person of ordinary skill in the art, and so forth.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person of ordinary skill in the art to which this invention belongs. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, suitable methods and materials are described below. All publications, patent applications, patents and other references discussed above are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the invention is not entitled to antedate such disclosure by virtue of its prior invention. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety including all figures and drawings.

Prior to setting forth the invention it may be helpful to an understanding thereof to set forth definitions of certain terms to be used hereinafter.

Activity: A function or set of functions performed by a molecule in a biological context. For e.g. human GM-CSF, biological activity is characterized by the stimulation of the proliferation and differentiation of certain

hematopoietic progenitor cells (e.g. TF1 or dendritic cell precurors). Human GM-CSF stimulates the formation of neutrophilic, eosinophilic, monocytic and megakaryocytic cells, as well as erythroid cells in the presence of erythropoietin. Higher activity in sense of the invention also means a favorable activity in sense of it biological and/or pharmaceutical meaning. For example, a glycoprotein which biological activity is increased to an extend by decreasing advers biological effects, for example decreased stimulation of adverse immune effects or decreased immunogenicity.

The novel glycoproteins of the present invention can be used in various clinical applications where the activity is desired These applications include chemotherapy, where recovery from cytotoxic drug-induced leukopenia may be speeded through the use of these proteins, which may allow more intensive use of such therapy. Treatment with these proteins may also permit more frequent use of myelotoxic drugs, speed recovery from bone marrow ablation during marrow transplantation and improve leukocyte production in states of marrow hyperproliferation, such as aplastic anemia. Furthermore, neutrophil production in persons being utilized as white blood cell donors may be enhanced. These proteins may also be used to enhance nonspecific host defense mechanisms in patients with overwhelming bacterial, fungal or parasitic infections, or in patients with non-responsive cancers. Certain proteins of the present invention are even more advantageous over the naturally-occurring GM-CSF due to their higher specific activities. This enhanced activity may allow the use of less material

per patient per dose, which can be expected to reduce undesirable side effects, such as capillary leak syndrome, which has been observed with therapeutic use of recombinant native GM-CSF.

By the term "regulating the immune response" or grammatical equivalents, herein is meant any alteration in any cell type involved in the immune response. The definition is meant to include an increase or decrease in the number of cells, an increase or decrease in the activity of the cells, or any other changes which can occur within the immune system. The cells may be, but are not limited to, T lymphocytes, B lymphocytes, natural killer (NK) cells, macrophages, eosinophils, mast cells, dendritic cells or neutrophils. The definition encompasses both a stimulation or enhancement of the immune system to develop a sufficiently potent response to a deleterious target, as well as a suppression of the immune system to avoid a destructive response to a desirable target. In the case of stimulation of the immune system, the definition includes future protection against subsequent tumor challenge.

By the term "cytokine" or grammatical equivalents, herein is meant the general class of hormones of the cells of the immune system, both lymphokines and monokines, and others. The definition is meant to include, but is not limited to, those hormones that act locally and do not circulate in the blood, and which, when used in accord with the present invention, will result in an alteration of an individual's immune response. The cytokine can be, but is not limited to, IL-2, IL-4, IL-6, IL-7, GM-CSF, gamma-IFN, TNF-alpha,

CD2 or ICAM. Additionally, cytokines of other mammals with substantial homology to the human forms of IL-2, GM-CSF, TNF-alpha, and others, will be useful in the invention when demonstrated to exhibit similar activity on the immune system. Similarly, proteins that are substantially analogous to any particular cytokine, but have relatively minor changes of protein sequence, will also find use in the present invention. It is well known that some small alterations in protein sequence may be possible without disturbing the functional abilities of the protein molecule, and thus proteins can be made that function as cytokines in the present invention but differ slightly from current known sequences. Finally, the use of either the singular or plural form of the word "cytokine" in this application is not determinative and should not limit interpretation of the present invention and claims. In addition to the cytokines, adhesion or accessory molecules or combinations thereof, may be employed alone or in combination with the cytokines. CSF refers to a family of lymphoicines which induce progenitor cells found in the bone marrow to differentiate into specific types of mature blood cells. The particular type of mature blood cell that results from a progenitor cell depends upon the type of CSF present. For instance, erythropoietin is believed to cause progenitor cells to mature into erythrocytes while thrombopoietin is thought to drive progenitor cells along the thrombocytic pathway. Similarly, granulocyte-macrophage colony formation is dependent on the presence of GM-CSF.

For administration to patients, the purified glycoproteins of the present invention are mixed with a pharmaceutically

acceptable carrier or diluent in accordance with routine procedures. Therapeutic formulations will be administered by intravenous infusion or by subcutaneous injection. The formulations can also contain, if desired, other therapeutic agents. Dosage levels of the order of from about 0.5 μg to about 150 mg per kilogram of body weight per: day are useful in the treatment of the above-indicated conditions. For example, inflammation may be effectively treated by the administration of from about 0.1 μg to 50 mg of the compound per kilogram of body weight per day. The effective amount of GM-CSF administered is from 0.1 to 500 μg of GM-CSF per kilogram of body weight. More preferably, the effective amount administered is from 1 μg to 100 μg and most preferably from 5 to 50 μg of GM-CSF per kilogram of body weight. The amount, frequency and period of administration will vary depending upon factors such as the level of the specific antibody titers or the class of antibody to be induced. The amount of active ingredient that may be combined with the carrier materials to produce a single dosage form will vary depending upon the host treated and the particular mode of administration. For example, a formulation intended for the oral administration of humans may vary from about 5 to about 95% of the total composition. Dosage unit forms will generally contain between from about 1 mg to about 500 mg of active ingredient. It will be understood, however, that the specific dose level for any particular patient will depend upon a variety of factors including the activity of the specific compound employed, the age, body weight, general health, sex, diet time of administration, route of

administration, rate of excretion, drug combination and the severity of the particular disease undergoing therapy.

In its first aspect the present invention provides a ready source of glycoprotein having a higher or lower activity than native glycoproteins using recombinant method comprising expression of the glycoprotein in a cell with a defect in the sugar nucleotide biosynthesis pathway of sialic acid, whereby the cells are cultured in a media comprising sialic acid intermediates and/or an another glycoproteins carrying sialic acid.

Briefly, a vector comprising a nucleotide sequence encoding the glycoprotein of the invention, and said vector is introduced into the cells with a defect in the sugar nucleotide biosynthesis pathway of sialic acid by methods commonly known in the art, for example, lipofection, electroporation, Ca-Phosphate-transfection and the like. Nucleotide sequence refers to a heteropolymer of deoxyribonucleotides. DNA sequences encoding the proteins provided by this invention may be assembled from cDNA fragments and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic gene that is capable of being expressed in a recombinant transcriptional unit. The preferred host-vector system for the isolation of a glycoprotein clone is based on expression of the cDNA of glycoprotein in a suitable transformation vector; such a vector may be, e.g., a plasmid, cosmid, virus, phagemide, bacteriophage or another vector used e.g. conventionally in genetic engineering or in transfection of mammal cells and may

comprise further genes such as marker genes which allow for the selection of said vector in a suitable host cell and under suitable conditions. Said vector may be one selected from commercially available vectors. Nonlimiting examples include plasmid vectors compatible with mammalian cells, such as pUC, pBluescript (Stratagene), pET (Novagen), pREP (Invitrogen), pCRTopo (Invitrogen), pcDNA3 (Invitrogen), pCEP4 (Invitrogen), pMC1 neo (Stratagene), pXT1 (Stratagene), pSG5 (Stratagene), EBO-pSV2neo, pBPV-1, pdBPVMMTneo, pRSVgpt, pRSVneo, pSV2-dhfr, pUCTag, pIZD35, pLXIN and pSIR (Clontech) and pIRES-EGFP (Clontech). For vector modification techniques, see Sambrook and Russel "Molecular Cloning, A Laboratory Manual", Cold Spring Harbor Laboratory, N.Y. (2001). Vectors can contain one or more replication and inheritance systems for cloning or expression, one or more markers for selection in the host, e.g., antibiotic resistance, and one or more expression cassettes.

These vectors can be synthesized by techniques well known to those skilled in this art. The components of the vectors such as enhancers, promoters, and the like may be obtained from natural sources or synthesized as described above. Basically, if the components are found in DNA available in large quantity, e.g. components such as viral functions, or if they may be synthesized, e.g. polyadenylation sites, then with appropriate use of restriction enzymes large quantities of vector may be obtained by simply culturing the source organism, digesting its DNA with an appropriate endonuclease, separating the DNA fragments, identifying the DNA containing the element of interest and recovering same.

Ordinarily, a transformation vector will be assembled in small quantity and then ligated to a suitable autonomously replicating synthesis vector such as a procaryotic plasmid or phage.

An enhancer is a nucleotide sequence that can potentiate the transcription of a gene independent of the position of the enhancer in relation to the gene or the orientation of the sequence. The vectors herein may include enhancers. Enhancers are functionally distinct from promoters, but appear to operate in concert with promoters. Their function on the cellular level is not well understood, but their unique characteristic is the ability to activate or potentiate transcription without being position or orientation dependent. Promoters need to be upstream of the gene, while enhancers may be present upstream or 5' from the promoter, within the gene as an intron, or downstream from the gene between the gene and a polyadenylation site or 3' from the polyadenylation site. Inverted promoters are not functional, but inverted enhancers are. Enhancers are cis-acting, i.e., they have an effect on promoters only if they are present on the same DNA strand.

The cells comprising the vector are cultured in a media comprising sialic acid intermediates and/or an another glycoproteins carrying sialic acid. The media conditions influence the residual sialylation amount of the glycoprotein of the invention.

In one embodiment of the present invention, the human active glycoprotein is produced by a recombinant process in

a cell with the defect in the sugar nucleotide biosynthesis pathway of sialic acid, whereby the defect is a mutation selected from the group comprising a dehydrogenase-, a transketolase-, a transaldolase-, an isomerase-, a dehydrogenase-, and preferred an epimerase-defect (e. g. UDP-GlcNAc 2-Epimerase).

In another embodiment of the present invention, the glycoprotein is selected from the group comprising g-CSF, GM-CSF, FSH, antibodies and/or fragments thereof; examples of further suitable immunomodulatory cytokines include interferons (e.g., IFN-alpha, IFN-beta and IFN-gamma), interleukins (e.g., IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10 and IL-12), tumor necrosis factors (e.g., TNF-alpha and TNF-beta), erythropoietin (EPO), FLT-3 ligand, macrophage colony stimulating factor (M-CSF), granulocyte colony stimulating factor (G-CSF), and granuloc-macrophage colony stimulating factor (GM-CSF). The most preferred immunomodulatory cytokine is GM-CSF, such as human GM-CSF. An alternatively preferred immunomodulatory cytokine is IL-2.

In a further embodiment of the present invention, the cells are mammalian cells selected from the group comprising NM-F9, NM-D4, Percy6, CHO.

In another embodiment of the present invention, the sialic acid intermediate is ManNAc and the another glycoprotein is fetuin.

In a further embodiment of the present invention, the glycoprotein a semi-sialylated glycoprotein, whereby the glycoprotein has a high activity and serum-stability.

Furthermore, the present invention relates to pharmaceutical composition of the present invention for use in therapy, comprising a glycoprotein of the invention, and a pharmaceutically-acceptable diluent or carrier. In a preferred embodiment said pharmaceutical composition is a vaccine or vaccine-adjuvant. In accordance with the present invention the term vaccine composition relates to any composition which can be used as a vaccine. A vaccine means a therapeutic or prophylactic use of the pharmaceutical composition which induces an immune response. The forms or methods for manufacturing vaccine compositions according to the present invention are not particularly limited, and a composition in a desired form can be prepared by applying a single method available in the field of the art or methods in an appropriate combination. For the manufacture of a vaccine composition, aqueous media such as distilled water for injection and physiological saline, as well as one or more kinds of pharmaceutical additives available in the field of the art can be used. For example, buffering agents, pH adjusting agents, solubilizing aids, stabilizing agents, soothing agents, antiseptics and the like can be used, and specific ingredients thereof are well known to those skilled in the art. The vaccine composition can also be prepared as a solid preparation such as a lyophilized preparation, and then prepared as an injection by adding a solubilizing agent such as distilled water for injection before use. Depending upon the manner of introduction, the

compounds may be formulated in a variety of ways as discussed below. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt %. The vaccine composition may be administered alone or in combination with other treatments, i.e., radiation, or other chemotherapeutic agents or anti-cancer agents. In a preferred embodiment, the vaccine compositions are in a water-soluble form, such as pharmaceutically acceptable salts, which is meant to include both acid and base addition salts. The vaccine compositions can be prepared in various forms, such as injection solutions, suspensions, and the like. The vaccine compositions may also include one or more of the following: carrier proteins such as serum albumin; buffers; stabilizing agents; colouring agents and the like. Additives are well known in the art, and are used in a variety of formulations. In addition, the glycoprotein of the invention, e.g. GM-CSF, will typically be used as vaccine adjuvant to enhance the protection afforded by animal or human vaccines that are considered weak (i.e., provide diminished protection in terms of level, extent, and/or duration). Examples of such vaccines are bacterins such as Bordetella bacterin, Escherichia coli bacterins, Haemophilus bacterins, Leptospirosis vaccines, Moraxella bovis bacterin, Pasteurella bacterin and Vibrio fetus bacterin, pneumococcal vaccines and attenuated live or killed virus products or recombinant antigenic viral products such as hepatitis B, influenza A & B, bovine respiratory disease vaccine, infectious bovine rhinotracheitis, parainfluenza-3, respiratory syncytial virus, bovine virus diarrhea vaccine, equine influenza vaccine, feline leukemia vaccine, feline respiratory

disease vaccine rhinotracheitiscalicipneumonitis viruses, canine parovovirus vaccine, transmissible gastroenteritis vaccine, pseudorabies vaccine, and rabies vaccine. The glycoprotein as vaccine-adjuvant will normally be administered separately from the vaccine, although it may be administered in combination with the vaccine. When glycoprotein as vaccine-adjuvant is combined with the vaccine, the composition administered contains an immunogen that is effective in eliciting a specific response to a given pathogen or antigen, a pharmaceutically acceptable vaccine carrier and an immunopotentiating amount of glycoprotein. Administration of glycoprotein as vaccine-adjuvant can be subcutaneous, intravenous, parenteral, intramuscular, or any other acceptable method. Preferably, vaccine-adjuvant is administered prior to the administration of the vaccine and at the same site where the vaccine is to be administered. The formulations and pharmaceutical compositions contemplated by the above dosage forms can be prepared with conventional pharmaceutically acceptable excipients and additives, using conventional techniques. Other adjuvants may be administered either with the vaccine or together with the glycoprotein.

Furthermore, the present invention relates to a kit comprising a glycoprotein of the invention, and/or synthetic analogues, modifications and pharmacologically active fragments thereof and an information about the using of parts of the kit. In an preferred embodiment of the present invention, the kit is a kit for enhancing an immunogenic response of a mammal to antigens in a vaccine

comprising a container of a pharmaceutical composition of highly/higher active GM-CSF, EPO or FSH and a pharmaceutically acceptable carrier therefore; and a container of a pharmaceutical composition of a vaccine and a pharmaceutically acceptable carrier therefore.

Furthermore, the present invention relates also to a method for differential sialylation of glycoproteins, whereby a cell with a defect in the sugar nucleotide biosynthesis pathway of sialic acid is transformed with a nucleic acid encoding the glycoprotein, whereby the cells are cultured in a media supplemented with different degrees of sialic acid intermediates and/or glycoproteins carrying sialic acid. Recombinant proteins are an important class of therapeutics used, for example, to replace deficiencies in critical blood borne growth factors and to strengthen the immune system to fight cancer and infectious disease. One embodiment of the present invention is focused (i) to create differential sialylated proteins and therefore (ii) to create improved drugs that are more effective and safer than currently available treatments. High and highest active as well as low and lowest active differential sialylated proteins are obtainable by said method for differential sialylation of glycoproteins.

Furthermore, the present invention relates in an alternative embodiment to a method for the determination (i) of highly active glycoproteins or (ii) for the determination of production-conditions of the glycoproteins comprising the steps of production of different sialylation forms of the glycoprotein by the method for differential

sialylation of glycoproteins of the invention; a determination of activity of the glycoprotein in a bioassay suitable for determining the activity and/or a determination of the optimal concentration of sialic acid intermediates and/or other glycoproteins carrying sialic acid.

Furthermore, the present invention relates to the use of the glycoprotein of the invention for prophylactic and/or therapeutic treatment of diseases selected from the group comprising neonatal infections, Neutropenia, Cytopenia, AML and myelodysplastic syndromes, cancer, HIV and/or diseases of hemotopoietic systems. Another preferred embodiment is the use of glycoprotein for treatment of proliferative blood disorders, such as certain leukemias and anemias, and human glycoprotein of the invention could prove useful in achieving successful bone marrow transplantation following cancer chemotherapy. In a further embodiment the glycoprotein of the invention is combined with other glycoprotein e.g. erythropoietin, thrombopoetin, G-CSF, M-CSF and/or SCF. The combination of the glycoprotein produced by the method of the invention and an other glycoprotein is also useful as cocktail of different chemotherapeutic agents (e.g. alkylating agents, doxyrubicin, carboplatinum, cisplatinum, taxol, and other drugs) and combinations of very high doses of chemotherapy with restorative agents. The ability of glycoproteins to stimulate granulocyte and macrophage production indicated that pharmaceutical compositions having activity of human glycoprotein of the invention are clinically useful in situations where increased production of these cell types

is required. In particular, compositions of glycoprotein of the invention are useful clinically for the treatment of myelo-suppression caused by chemotherapeutical or irradiation treatment of cancer. The terms treating cancer, therapy, and the like refer generally to any improvement in the mammal having the cancer wherein the improvement can be ascribed to treatment with the compounds of the present invention. The improvement can be either subjective or objective. For example, if the mammal is human, the patient may note improved vigor or vitality or decreased pain as subjective symptoms of improvement or response to therapy. Alternatively, the clinician may notice decrease in tumor size or tumor burden based on physical exam, laboratory parameters, tumor markers or radiographic findings. Some laboratory signs that the clinician may observe for response to therapy include normalization of tests such as white blood cell count, red blood cell count, platelet count, erythrocyte sedimentation rate, and various enzyme levels. Additionally, the clinician may observe a decrease in a detectable tumor marker. Alternatively, other tests can be used to evaluate objective improvement such as sonograms, nuclear magnetic resonance testing and positron emissions testing.

In addition, glycoproteins of the invention are useful in treating severe infections because glycoproteins can increase and/or activate the number of granulocytes and/or monocytes. The glycoprotein of present invention can be used by any conventional method such as, for example, via parenteral, ocular, topical, inhalation, transdermal,

vaginal, buccal, transmucosal, transurethral, rectal, nasal, oral, pulmonary or aural routes.

In an preferred embodiment of the invention, the use of the glycoproteins in the context of the invention is a combined glycoprotein/radiotherapy, glycoprotein/chemotherapy and/or a immune-stimulation therapy. In another aspects of the preferred embodiment, the glycoproteins described herein may be used for immunotherapy of cancer. A cancer may be diagnosed using criteria generally accepted in the art, including the presence of a malignant tumor. Pharmaceutical compositions and immunogenic compositions may be administered either prior to or following surgical removal of primary tumors and/or treatment such as administration of radiotherapy or conventional chemotherapeutic drugs.

In another preferred embodiment of the invention, the glycoproteins are used for stimulating proliferation, development, differention and activitation of blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood or fetal liver. In particular, the glycoprotein stimulate the production, the development and the formation of colonies of granulocytes, macrophages, eosinophils and megakaryocytes. The glycoprotein induce in particular a macrophagic cytotoxocity, stimulates antibody-dependent cytotoxic activity (ADCC) and the recruitment of leukocytes at the level of the sites of inflammation.

Furthermore, the present invention relates to the use of NM-F9 and/or NM-D4 cells for producing glycoproteins of the invention. Surprisingly, it was found that the cell lines NM-F9 and NM-D4 which were derived from K562 cells (ATCC CCL-243) are useful for differential sialylation of glycoproteins. The term NM-F9 or NM-D4 cell relates to the specific cell-clones NM-F9 and/or NM-D4 as well as subclones thereof. The term subclones means cells or cells of a cell line which are derived from NM-F9 or NM-D4 and which occur due to naturally occurring alterations, e.g., mutations, but having similar characteristics as the above-mentioned cell lines.

Cell lines NM-F9 and NM-D4 were deposited on August 14, 2003 by Nemod Biotherapeutics GmbH & Co. KG, Robert-Rössle-Str. 10, 13125 Berlin (Germany) at the "DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH" in Braunschweig (Germany), which is an International Depositary Authority according to the Budapest Treaty. Cell line NM-F9 is deposited under DSM ACC2606 and cell line NM-D4 is deposited under DSM ACC2605.

As will be apparent to those skilled in the art in which the invention is addressed, the present invention may be embodied in forms other than those specifically disclosed above without departing from the spirit or essential characteristics of the invention. The particular embodiments of the present invention, described above, are therefore to be considered in all respects as illustrative and not restrictive. The scope of the present invention is

as set forth in the appended claims rather than being limited to the examples contained in the foregoing description.

The following figures 1 to 44 illustrate the glycoengineering of the invention for the production of highly active human proteins with an optimized sialylation.

Example

Generation of a sialylation variant of hGM-CSF with an increased bioactivity

The method is based on human glycoengineered cell lines NM-F9 and NM-D4 which are deficient in sialylation. These glycoengineered cell lines were generated from a rapidly dividing, virus-free human myeloid leukaemia cell line using random mutagenesis and phenotypic selection with a Thomsen-Friedenreich-specific antibody. TF-positive sialic acid deficient cell lines were single cell cloned, adapted to serum-free media and characterized in detail for their carbohydrate determinants and transferase expression. The capacity to sialylate could be reconstituted by addition of certain defined serum additives. NM-F9 was stably transfected with a potent expression vector encoding the human growth factor GM-CSF. High amounts of secretory GM-CSF could be produced. Addition of varying amounts of defined serum additives allowed the generation of various GM-CSF variants with differing sialylation degree. In vitro activity tests were performed using the TF1 proliferation test and a novel dendritic cell proliferation and

activation test comprising the standardized dendritic precursor cell line NMDC-11 (NemodDC) which originates from MUTZ-3. Both tests showed that a certain sialylation variant expressed a several fold increase in activity compared to hGM-CSF expressed in bacteria (Leukomax) and yeast (Leukine) as well as those variants with lower or higher sialylation degrees. The rather high degree of sialylation of the highly active hGM-CSF with fully human glycosylation leads to largely improved pharmacokinetic properties and bioactivity compared to standard hGM-CSF preparations.

Claims

1. Human active glycoprotein produced by a process comprising expression of the glycoprotein in a cell with a defect in the sugar nucleotide biosynthesis pathway of sialic acid, whereby the cells are cultured in a medium comprising sialic acid intermediates and/or other glycoproteins carrying sialic acid.

2. Glycoprotein according to claim 1,
characterized in that,
the defect is a mutation of a epimerase.

3. Glycoprotein according to claim 1 or 2,
characterized in that,
the glycoprotein is selected from the group comprising EPO, g-CSF, GM-CSF, FSH and/or fragments thereof.

4. Glycoprotein according to claims 1 - 3,
characterized in that,
the cells are mammalian cells selected from the group comprising NM-F9, NM-D4, Percy6, CHO.

5. Glycoprotein according to claims 1 - 4,
characterized in that,
the sialic acid intermediate is ManNAc and the other glycoprotein is fetuin.

6. Glycoprotein according to claims 1 - 5,
characterized in that,

the glycoprotein is a semi-sialylated glycoprotein having higher activity than a native glycoprotein.

7. Pharmaceutical composition for use in therapy, comprising a glycoprotein of claims 1 - 5, and a pharmaceutically-acceptable diluent or carrier.

8. Pharmaceutical composition according to claim 6, characterized in that,
the composition is a vaccine or vaccine-adjuvant.

9. Kit comprising a glycoprotein of claims 1 - 5, and/or synthetic analogues, modifications and pharmacologically active fragments thereof and an information about the using of parts of the kit.

10. Method for differential sialylation of glycoproteins, characterized in that,
a cell with a defect in the sugar nucleotide biosynthesis pathway of sialic acid is transformed with a nucleic acid encoding the glycoprotein, whereby the cells are cultured in a media supplemented with different degrees of sialic acid intermediates and/or glycoproteins carrying sialic acid, and a glycoprotein according claims 1- 6 is obtained.

11. Method for determination (i) of highly active glycoproteins, (ii) for determination of conditions for productions or (iii) for determination of serum-half-life of the glycoproteins
comprising the steps of

(a) production of different sialylation forms of the glycoprotein by the method according claim 10;

(b) determination of activity or serum-half-life of the glycoprotein in a bioassay suitable for determining the activity or serum-half-life and/or;

(c) determination of the optimal concentration of sialic acid intermediates and/or other glycoproteins carrying sialic acid.

12. Use of the glycoprotein according to claims 1 - 6 for prophylactic and/or therapeutic treatments of diseases selected from the group comprising bone marrow transplantation, Neutropenia, Cytopenia, AML and myelodysplastic syndromes, cancer, HIV and/or diseases of hemotopoietic systems.

13. Use according to claim 12, characterized in that, the glycoprotein is combined with erythropoietin, thrombopoetin, G-CSF, M-CSF and/or SCF.

14. Use according to claim 12 or 13, characterized in that, the use is via parenteral, ocular, topical, inhalation, transdermal, vaginal, buccal, transmucosal, transurethral, rectal, nasal, oral, pulmonary or aural routes.

15. Use according to claims 12 - 14, characterized in that, the use is a combined glycoprotein/radiotherapy, glycoprotein/chemotherapy and/or a immune-stimulation therapy.

16. Use of the glycoprotein according to claims 1 - 6 for stimulating proliferation, development, differention and activitation of granulocytes, macrophages, eosinophils and their progentior cells.

17. Use of a method according to claims 10 or 11 for producing glycoproteins according to claims 1 - 6 with an optimized serum-half-life, pharmacokinetics and/or immunogenicity.

18. Use of NM-F9 and/or NM-D4 cells for producing glycoproteins according to claims 1 - 6.

Abstract

The present invention relates to human active glycoproteins, a pharmaceutical composition for use in therapy comprising the glycoproteins, a method for differential sialylation of the glycoproteins, a method for the determination (i) of highly active glycoproteins and for the determination (ii) of production conditions of the glycoproteins, and the invention relates to the use of the glycoproteins for prophylactic and/or therapeutic treatment of diseases, particularly bone marrow transplantation, Neutropenia, Cytopenia, AML and myelodysplastic syndromes, cancer, HIV and/or diseases of hemotopoietic systems.

Glycosylation is important

- Activity
- Serum half-life
- Stability
- Immunogenicity

Fig. 1

GLYCOTOPE

Aim

Generation of an expression platform

which

allows a fully human glycosylation

&

with an optimized sialylation degree

Fig. 2

GLYCOTOPE

First Step

Generation of a sialylation deficient cell line

which is highly TF-positive

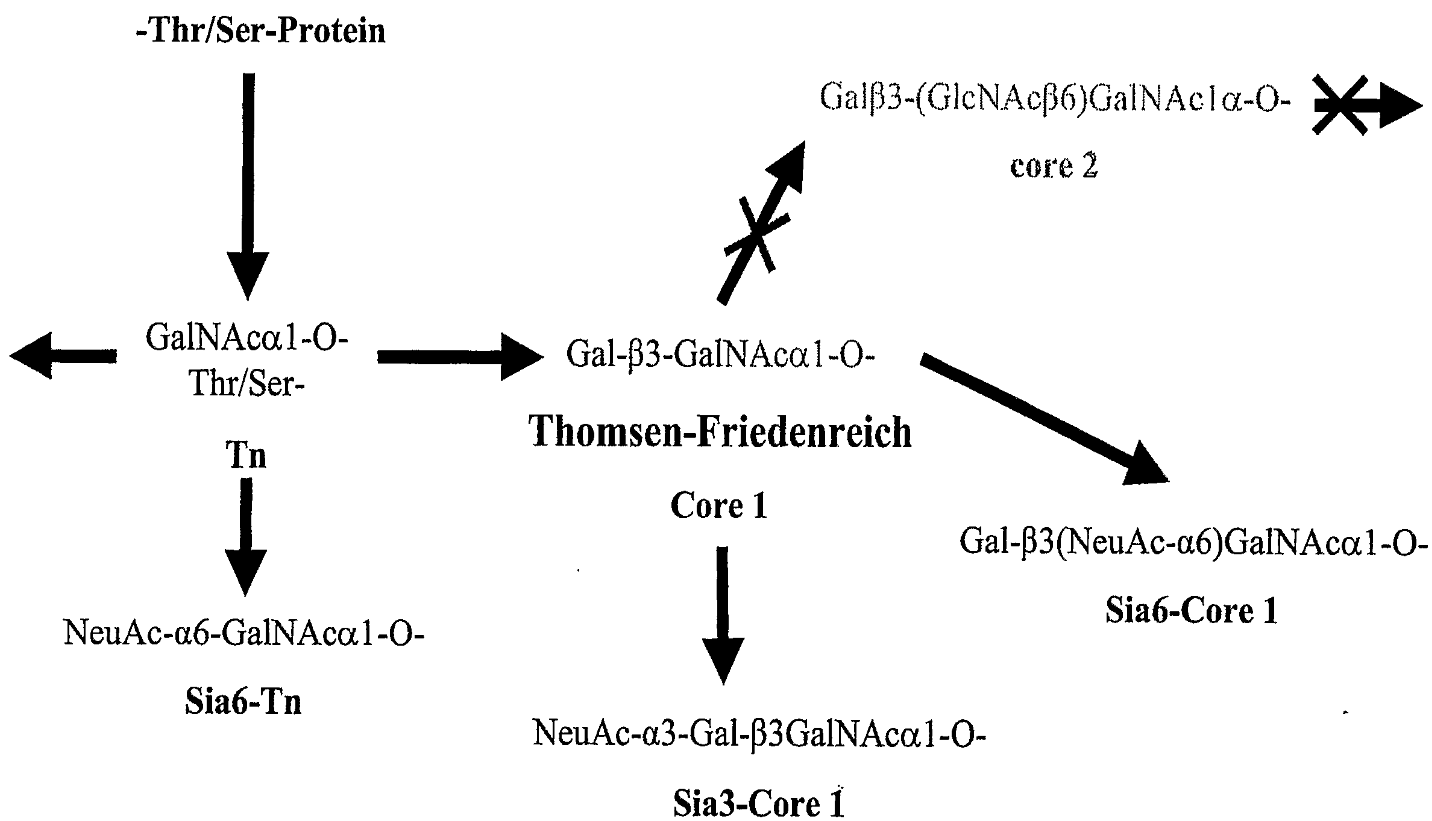
by

Cellular GlycoEngineering

Fig. 3

GLYCOTOPE

Why TF



- Defined CH structure which is masked by α2-3 and α2-6 linked sialic acids
- TF exposure is expected to need a defect in several sialyltransferases, sugar nucleotide biosythesis pathway or transproters

Requirement: biotechnologically suitable cell line deficient in core-2 elongation and with cryptic TF

Fig. 4

GLYCOTOPE

Strategy: Cellular GlycoEngineering

GlycoProfiling: Characterization of glycosyltaion profile of candidate cell lines for selection of suitable cell lines

↓

GlycoEngineering: Cellular GlycoEngineering

↓

GlycoAnalytics: Detailled characterization and selection of modified cell lines

↓

Differential Sialylation by metabolic engineering

Fig. 5

GLYCOTOPE

Methods for GlycoProfiling

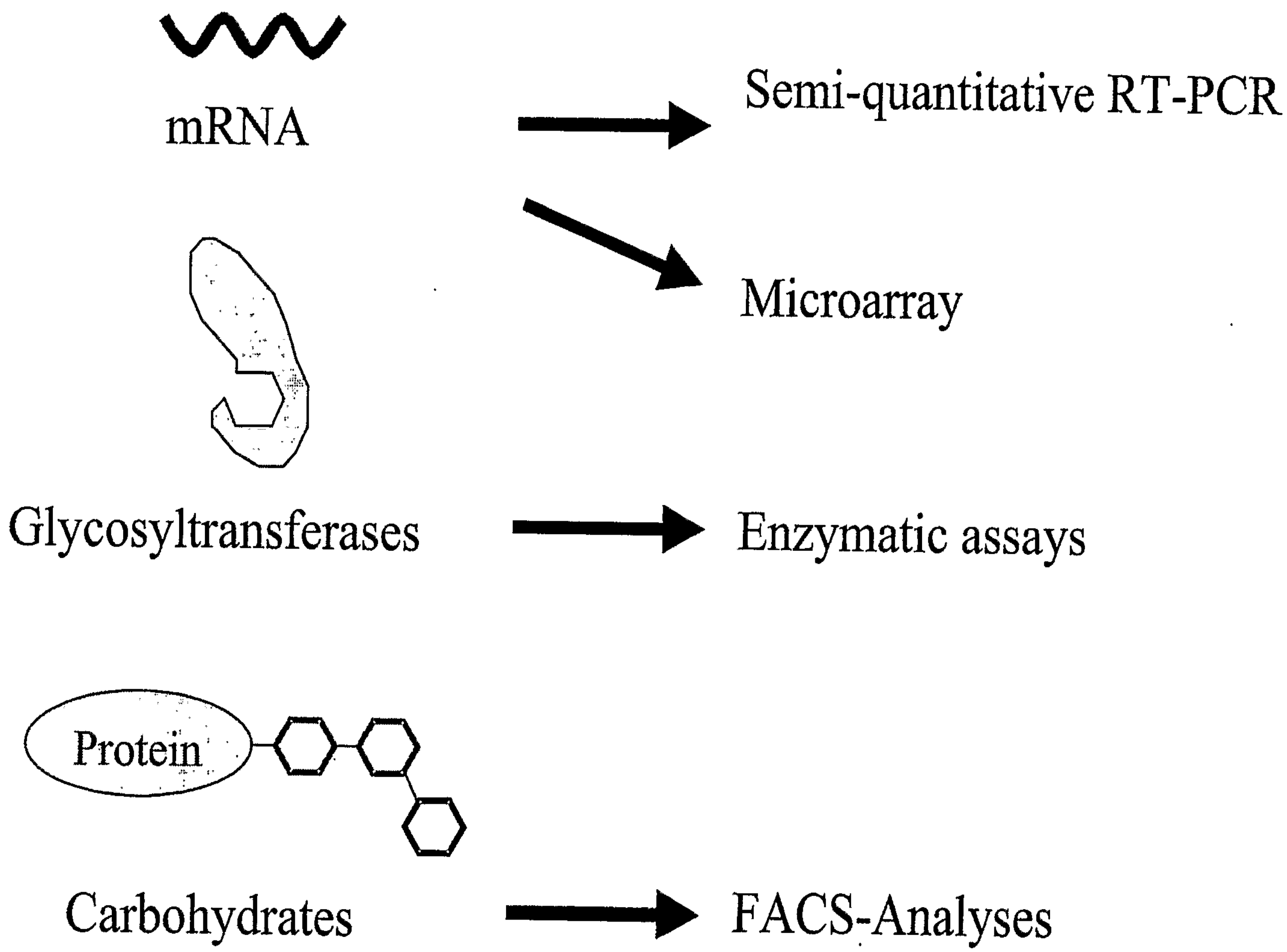


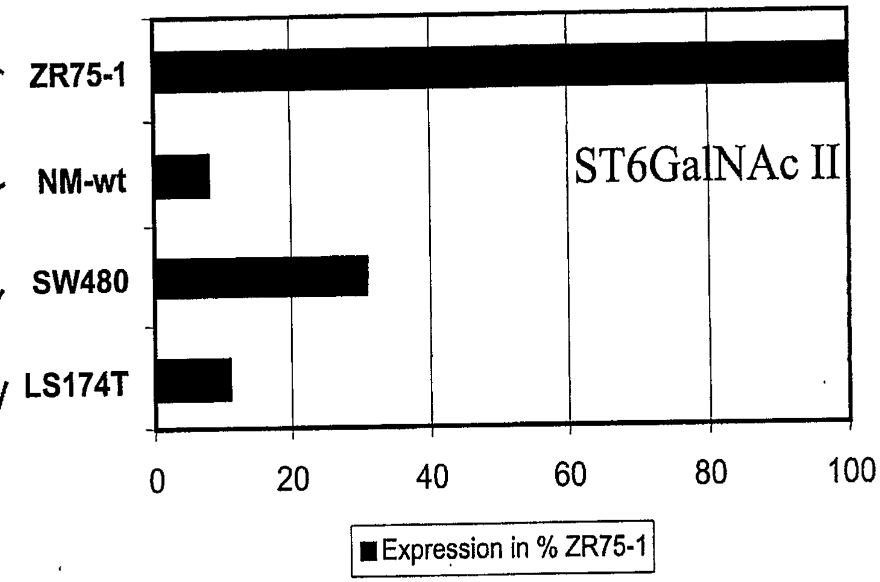
Fig. 6

GLYCOTOPE

GlycoProfiling: Expressionprofiles of glycosyltransferases differ greatly in human cell lines (I)

RT-PCR

		Expression in % ZR75-1
ZR75-1 (Standard)	ST3Gal I	100
	ST3Gal II	100
	ST6GalNAc I	100
	C2GNT L	100
	C2GNT M	100
	C1T1	100
	ST6Gal I	100
NM-wt	ST3Gal I	32
	ST3Gal II	117
	ST6GalNAc I	93
	C2GNT L	0
	C2GNT M	0
	C1T1	71
	ST6Gal I	109
SW480	ST3Gal I	29
	ST3Gal II	38
	ST6GalNAc I	0
	C2GNT L	9
	C2GNT M	0
	C1T1	36
	ST6Gal I	32
LS174T	ST3Gal I	108
	ST3Gal II	48
	ST6GalNAc I	69
	C2GNT L	15
	C2GNT M	282
	C1T1	48
	ST6Gal I	29



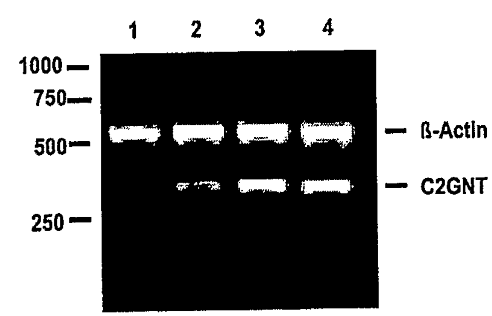


Fig. 7

GLYCOTOPE

GlycoProfiling: Expressionprofiles of glycosyl-transferases differ greatly in human cell lines (II)

Glycosyl-transferase	ZR75-1	HEK293	NM-wt	KG1	LS174T	MCF-7	SW480	T47D
C1GalT1	100	102	71	105	48	101	36	84
C2GNT-L	100	91	0	10	15	64	9	0
C2GNT-M	100	50	0	0	282	63	0	188
ST6GalNAc-I	100	43	93	11	69	18	0	3
ST6GalNAc-II	100	38	8	0	11	80	31	83
ST3Gal-I	100	53	32	73	108	87	29	64
ST3Gal-II	100	120	117	76	48	90	38	70
ST6Gal-I	100	94	109	72	29	40	32	83

[a] **Relative values in percentage of that of ZR75-1.**

Duplex RT-PCR used for determination of the glycosyltransferase vs. housekeeping gene ß-actin expression in each case.

Fig. 8

GLYCOTOPE

GlycoProfiling: Expressionprofiles of glycosyltransferases differ greatly in human cell lines (II)

Glycosyl-transferase	ZR75-1	HEK293	NM-wt	KG1	LS174T	MCF-7	SW480	T47D
C1GalT1	100	102	71	105	48	101	36	84
C2GNT-L	100	91	0	10	15	64	9	0
C2GNT-M	100	50	0	0	282	63	0	188
ST6GalNAc-I	100	43	93	11	69	18	0	3
ST6GalNAc-II	100	38	8	0	11	80	31	83
ST3Gal-I	100	53	32	73	108	87	29	64
ST3Gal-II	100	120	117	76	48	90	38	70
ST6Gal-I	100	94	109	72	29	40	32	83

[a] **Relative values in percentage of that of ZR75-1.**

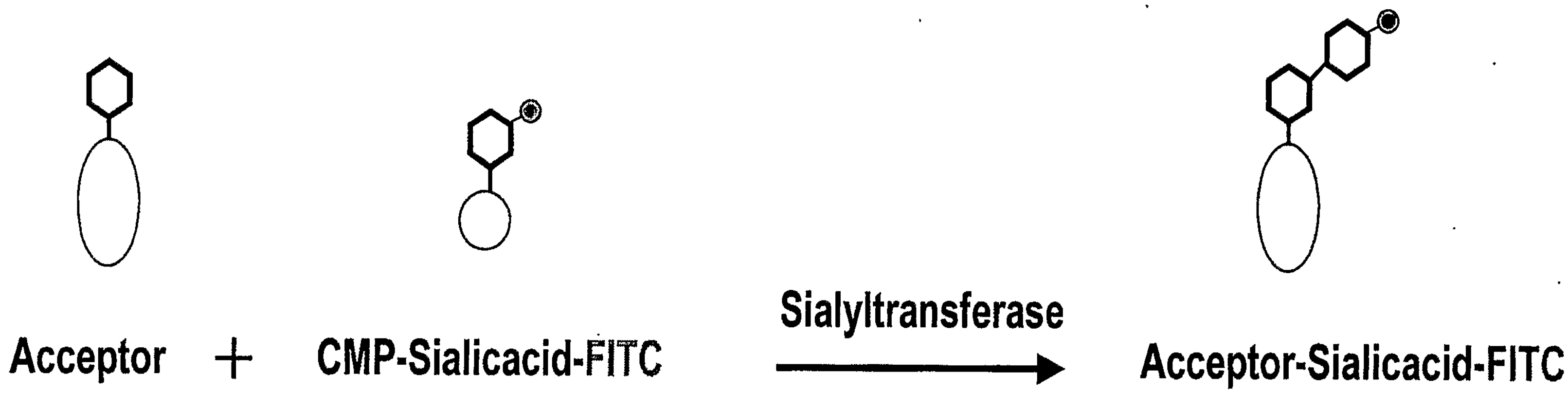
Duplex RT-PCR used for determination of the glycosyltransferase vs. housekeeping gene ß-actin expression in each case.

Fig. 9

GLYCOTOPE

GlycoProfiling: Sialyltransferase-Enzyme-Assay

Determination of Sialyltransferaseactivity



- Separation of reaction product via gelchromatography
- Quantification via measurement of fluorescence intensity

Fig. 10

GLYCOTOPE

Acceptors determine the specificity of the assay

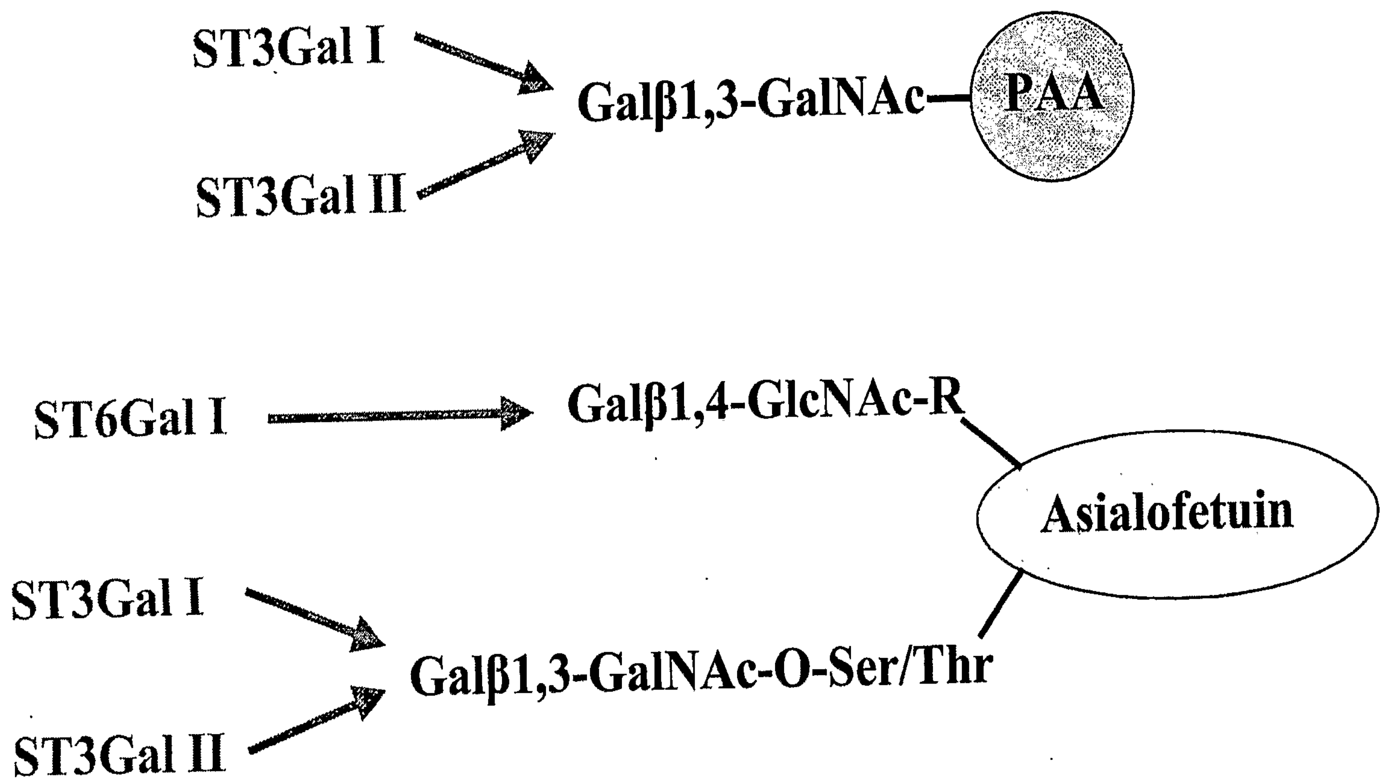


Fig. 11

GLYCOTOPE

NM-wt shows highest activity for sialylation of polylactosamines and medium activity for TF-sialylation

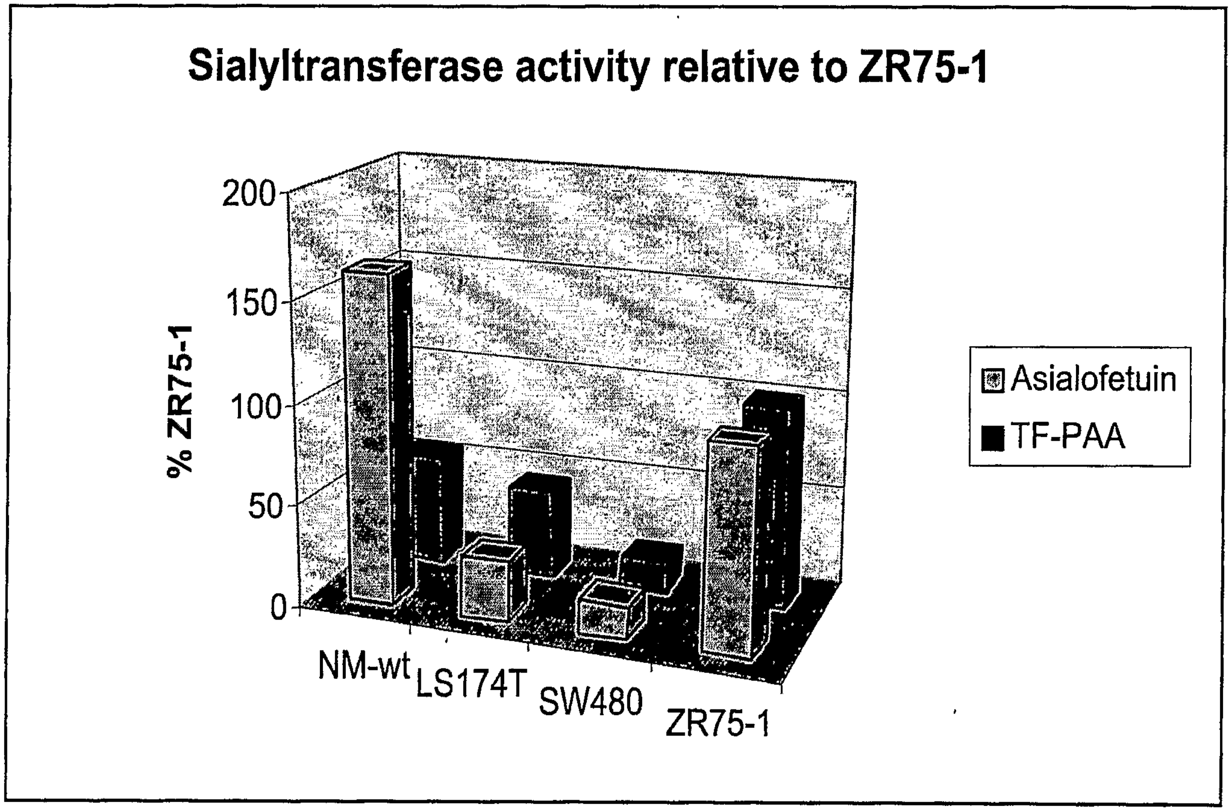


Fig. 12

GLYCOTOPE

GlycoProfiling: Carbohydrate Determinants

MAb/ lectin	HEK293	NM-wt	LS174T	MCF-7	SW480	T47D	ZR75-1
A78-G/A7	0	1	0	1	1	1	9
A78-G/A7	9	40	2	4	11	4	20
PankoMab	0	3	0	2	0	6	24
A83-C/B12	0	9	0	0	0	1	0
PNA	0	2	0	1	4	2	30
PNA	7	41	1	13	19	76	343
Jacalin	6	8	5	11	6	74	35
Jacalin	15	151	6	43	13	229	187
ACA	3	53	1	31	48	19	71
ACA	9	85	1	35	60	52	125
BPL	5	3	0	8	9	2	22
BPL	18	64	1	35	50	82	200

Untreated cells

Desialinated cells (Neuramidase)

Fig. 13

GLYCOTOPE

GlycoProfiling: Carbohydrate Determinants

High amounts of cryptic TF

MAb/ lectin	HEK293	NM-wt	LS174T	MCF-7	SW480	T47D	ZR75-1
A78-G/A7	0	1	0	1	1	1	9
A78-G/A7	9	40	2	4	11	4	20
PankoMab	0	3	0	2	0	6	24
A83-C/B12	0	9	0	0	0	1	0
PNA	0	2	0	1	4	2	30
PNA	7	41	1	13	19	76	343
Jacalin	6	8	5	11	6	74	35
Jacalin	15	151	6	43	13	229	187
ACA	3	53	1	31	48	19	71
ACA	9	85	1	35	60	52	125
BPL	5	3	0	8	9	2	22
BPL	18	64	1	35	50	82	200

Untreated cells (shaded) — Desialinated cells (Neuramidase) (white)

Fig. 14

GLYCOTOPE

Selection of NM-wt for TF-GlycoEngineering

Combination of aspects:

- Lack of expression of Core 2 enzymes C2GNT-L and C2GNT-M
- Highest expression of ST6Gal
- High expression of at least one ST3Gal, ST6GalNAc and ST6Gal transferase
- Highest sialytransferase activities for N-glycan substrate
- Highest amount of cryptic TF
- Grows in suspension with a high doubling rate of 24 h
- Single-cell cloning is easy
- Virus free.

Fig. 15

GLYCOTOPE

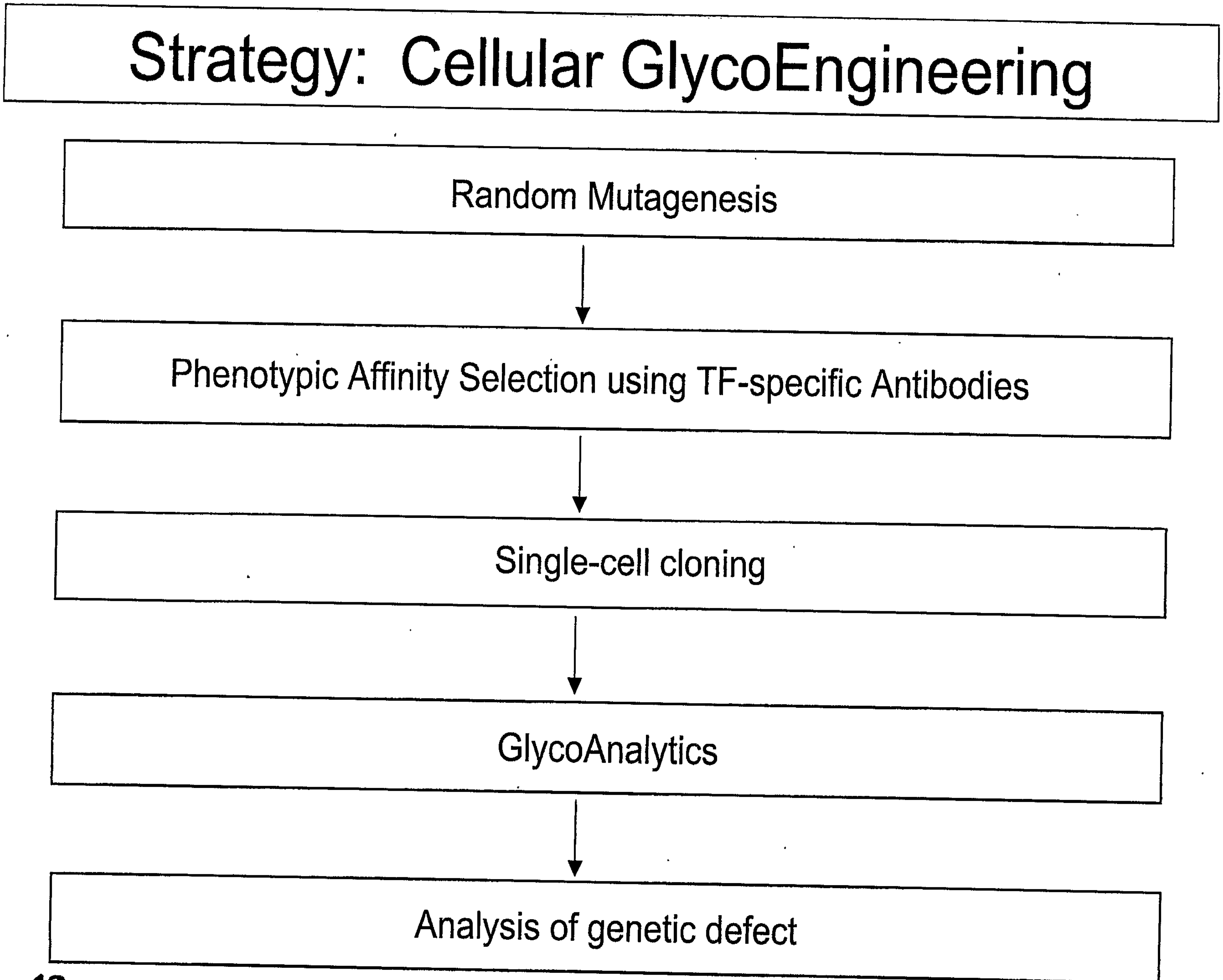


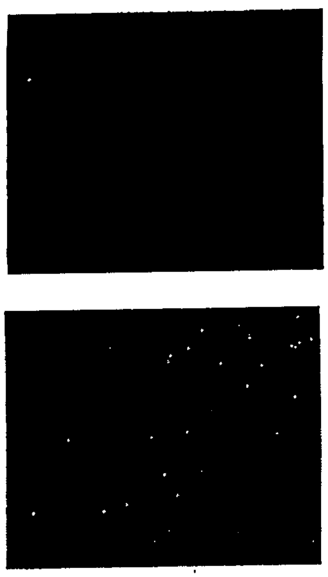
Fig. 16

GLYCOTOPE

Glycoengineered human NM-F9 cells are largely reduced in sialylation

Glycoengineering

EMS-treated origin cells

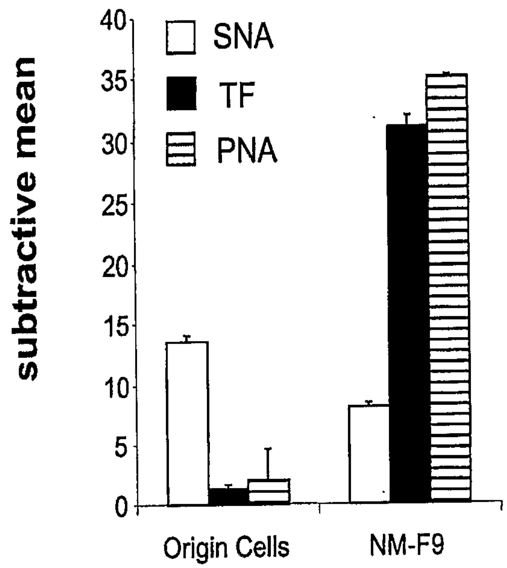


Exposure of desialylated epitopes

Affinity selected and cloned NM-F9

Random mutagenesis & Phenotypic selection

Determination of sialylation degree



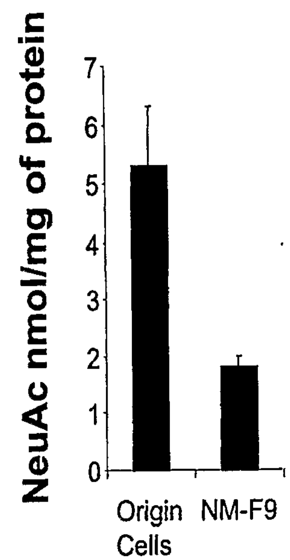
Flow cytometry

TF-specific antibody (A78-G/A7)

terminal β-Gal recognizing lectin (PNA)

sialic acid specific lectin (SNA).

(subtractive mean = (mean mab /lectin – mean isotype mab)



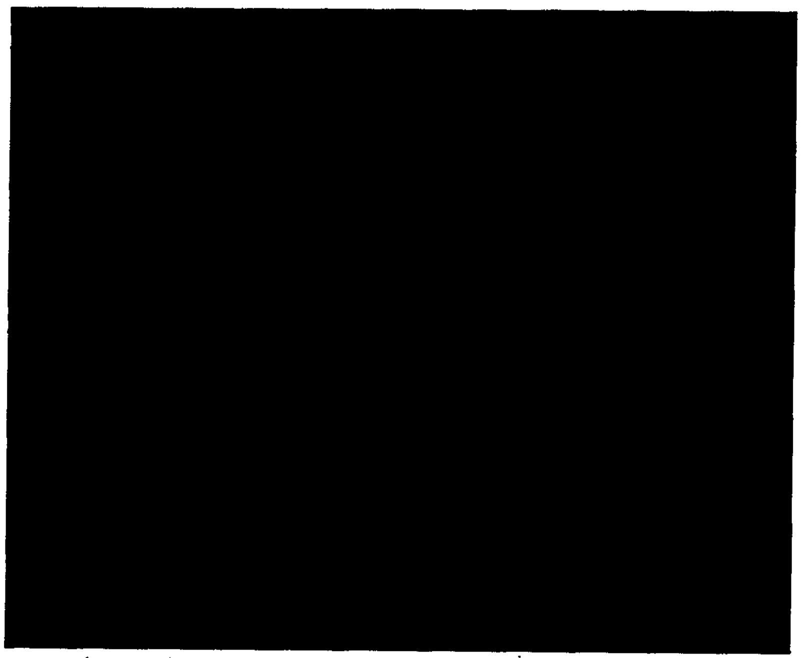
Thiobarbituric acid method

Fig. 17

GLYCOTOPE

Generation of TF high expressing cell lines

K562 before affinity selection



NM-F9

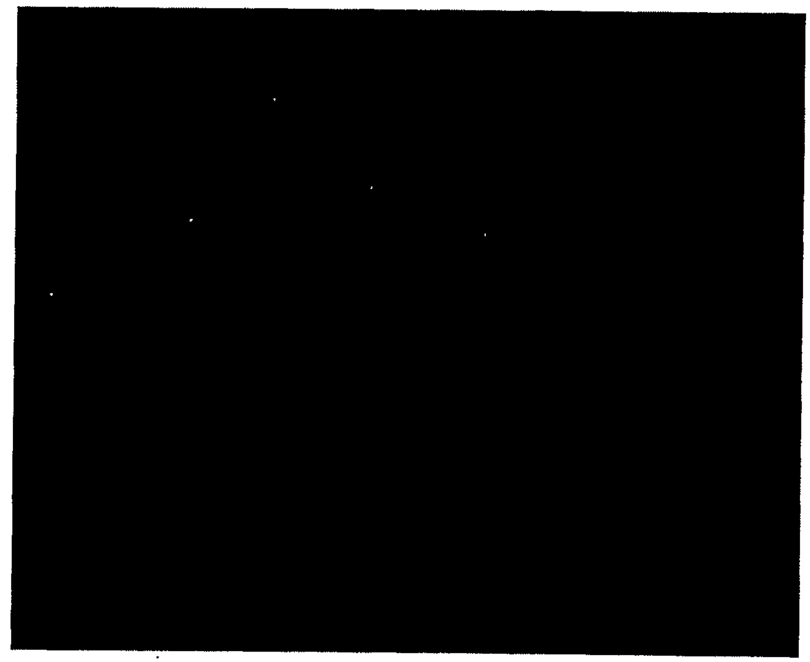


Fig. 18

GlycoAnalytics (I): TF high expressing cell lines express also asiologlycophorin, Le^{X} and Tn

Flow Cytometry

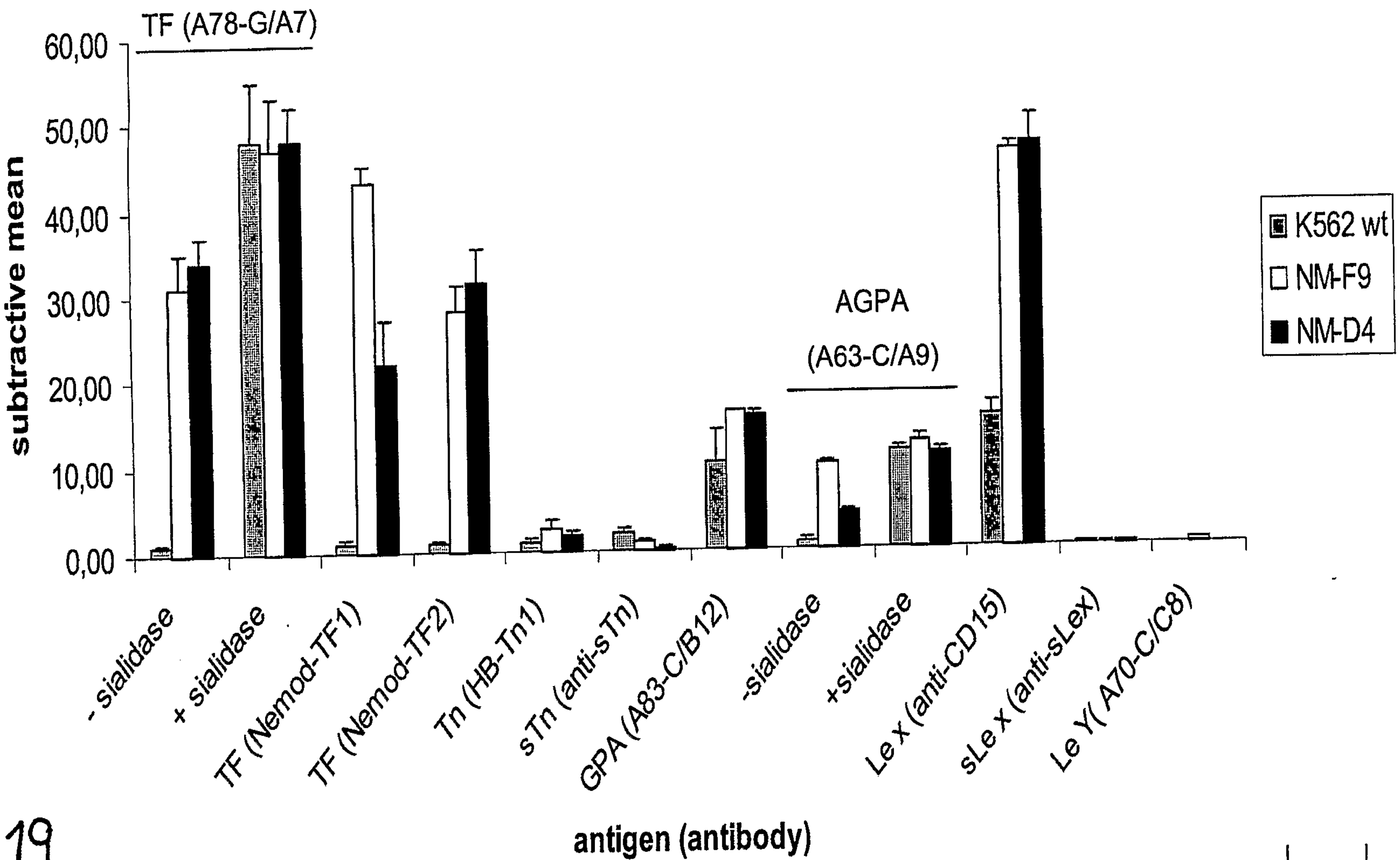


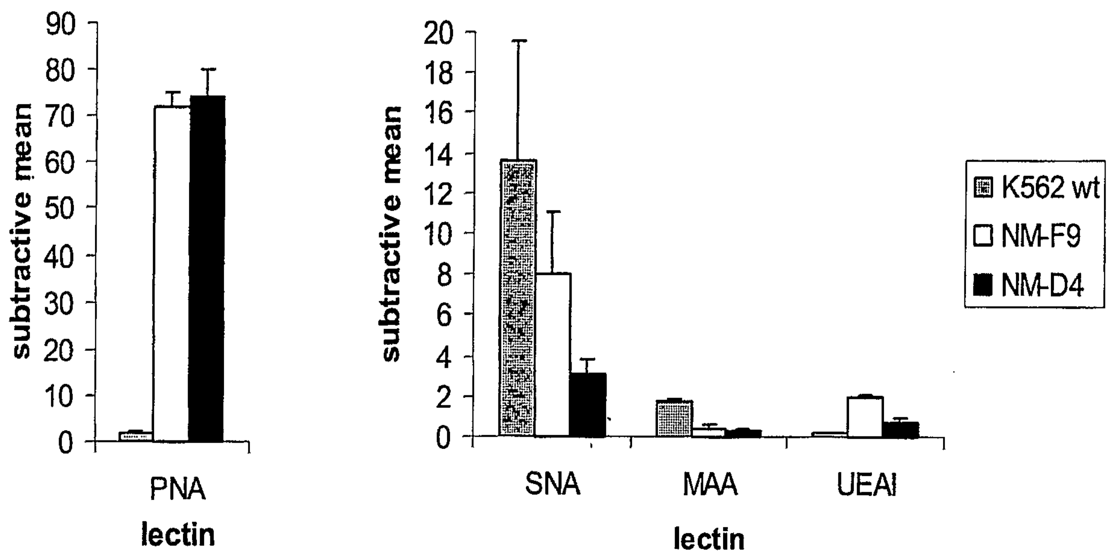
Fig. 19

GLYCOTOPE

GlycoAnalytics (II):
of TF high expressing cell lines

Sialic Acids

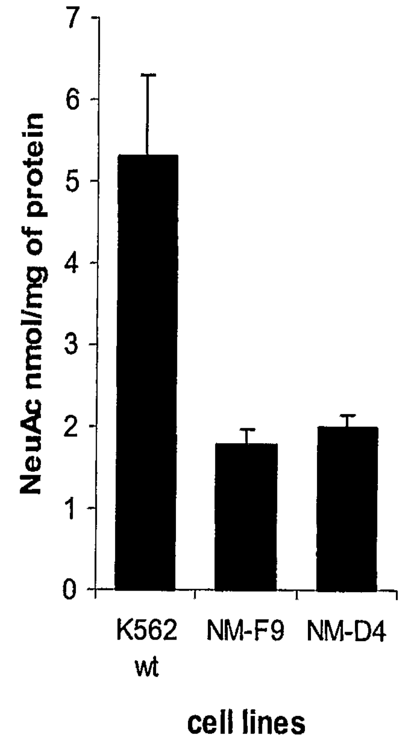
Flow Cytometry



⇒ **α2-3 sialic acids are absent and α2-6 sialic acids are reduced**

(media with serum)

Sialic acid content



Determination of membrane glycoconjugate-bound sialic acid by thiobarbituric acid method

GLYCOTOPE

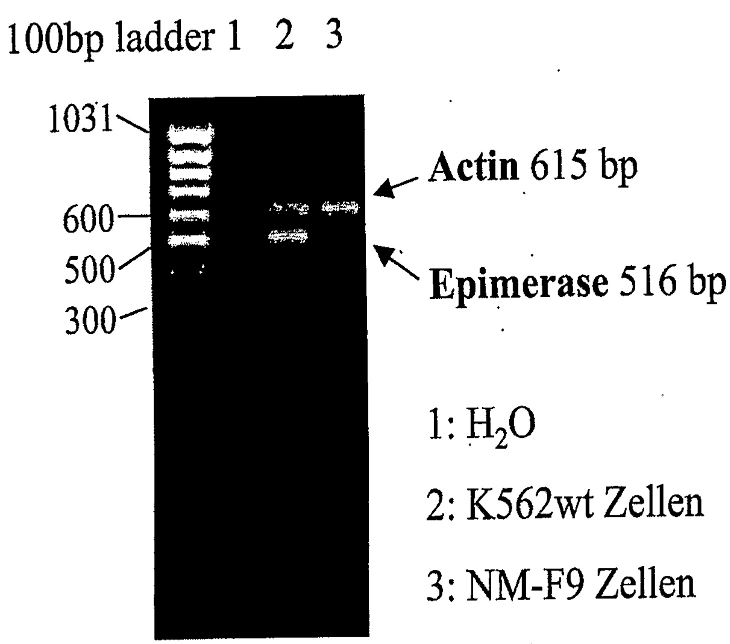
Fig. 20

Analysis of the genetic defect

UDP-GlcNAc 2-Epimerase

UDP-N-Acetylglucosamine $\not\longrightarrow$ N-Acetylmannosamine

mRNA expression analysis of UDP-GlcNAc 2-Epimerase



RT-PCR

Enzyme activity analysis of UDP-GlcNAc 2-Epimerase

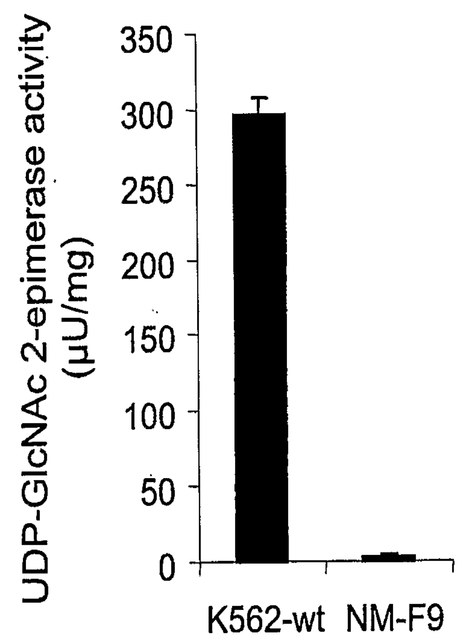
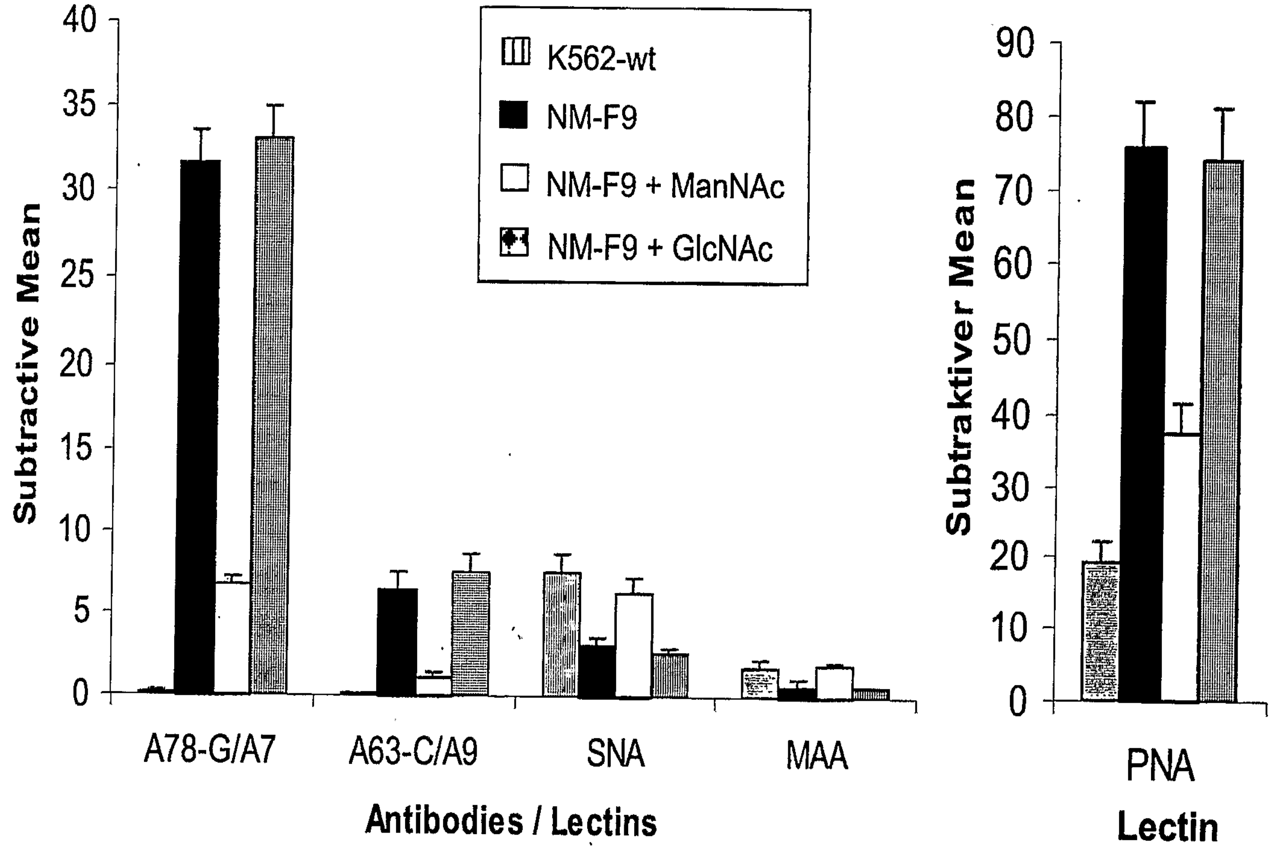


Fig. 21

GLYCOTOPE

Reconstitution of Sialylation by Metabolic Complementation

Flow Cytometry



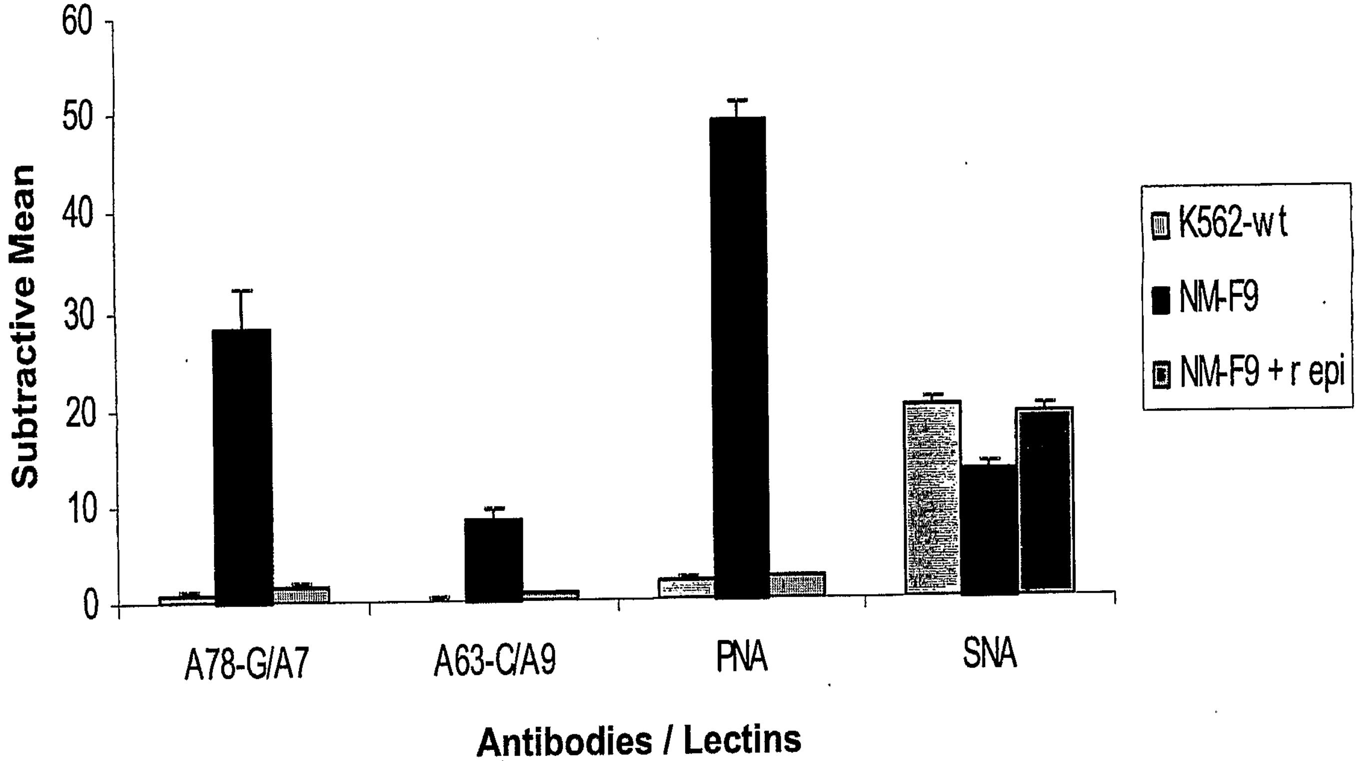
NM-F9 and origin cell cultivated for 4 days serum containing + / - 50 mM N-Acetylmannosamine or N-Acetylglucosamine

Fig. 22

GLYCOTOPE

Reconstitution of Sialylation by Genetic Complementation

Flow Cytometry



NM-F9 + r epi: NM-F9 cells stably transfected with the epimerase encoding construct pcDNA3.1Zeo(-)/2Epi

Fig. 23

GLYCOTOPE

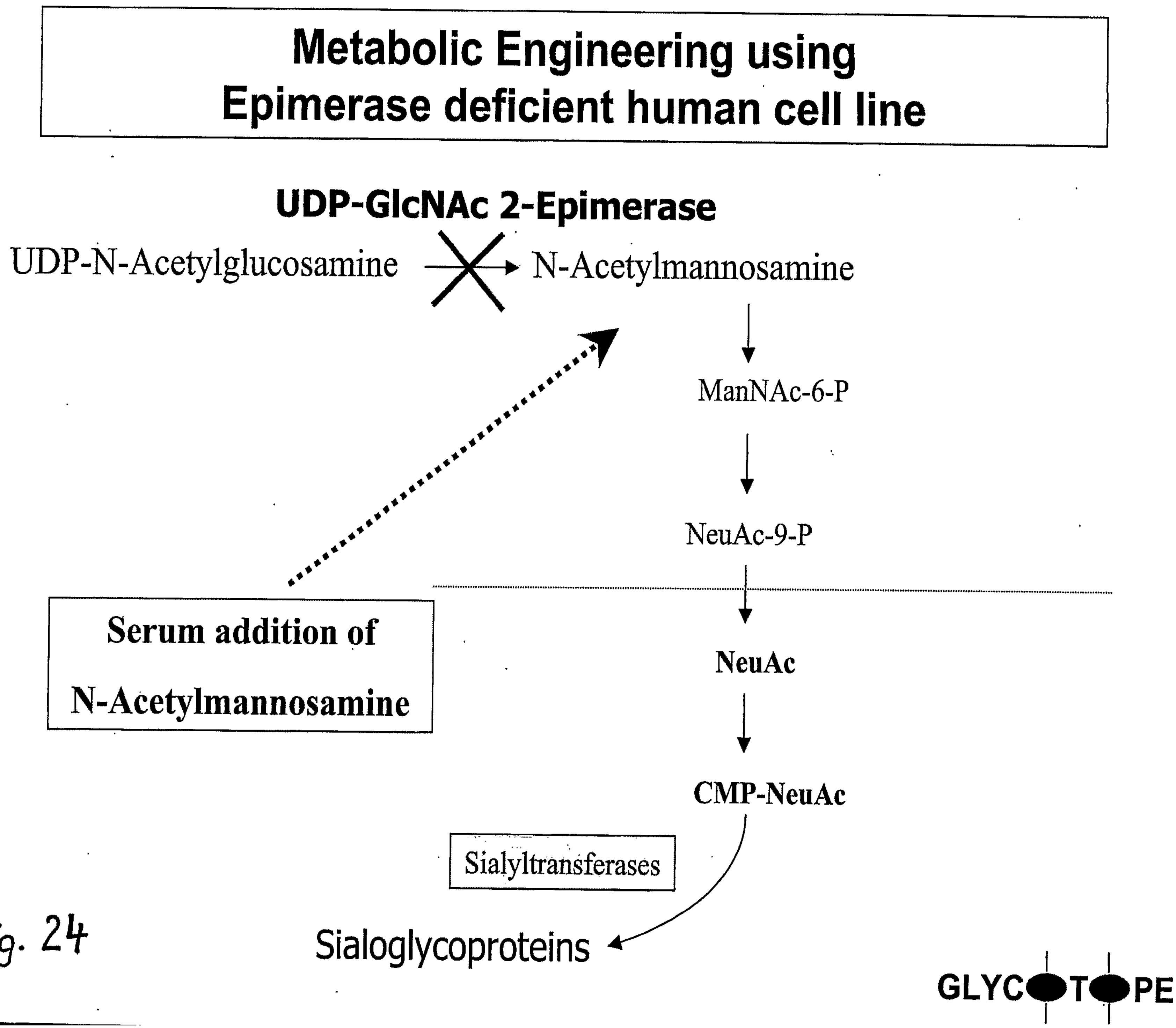
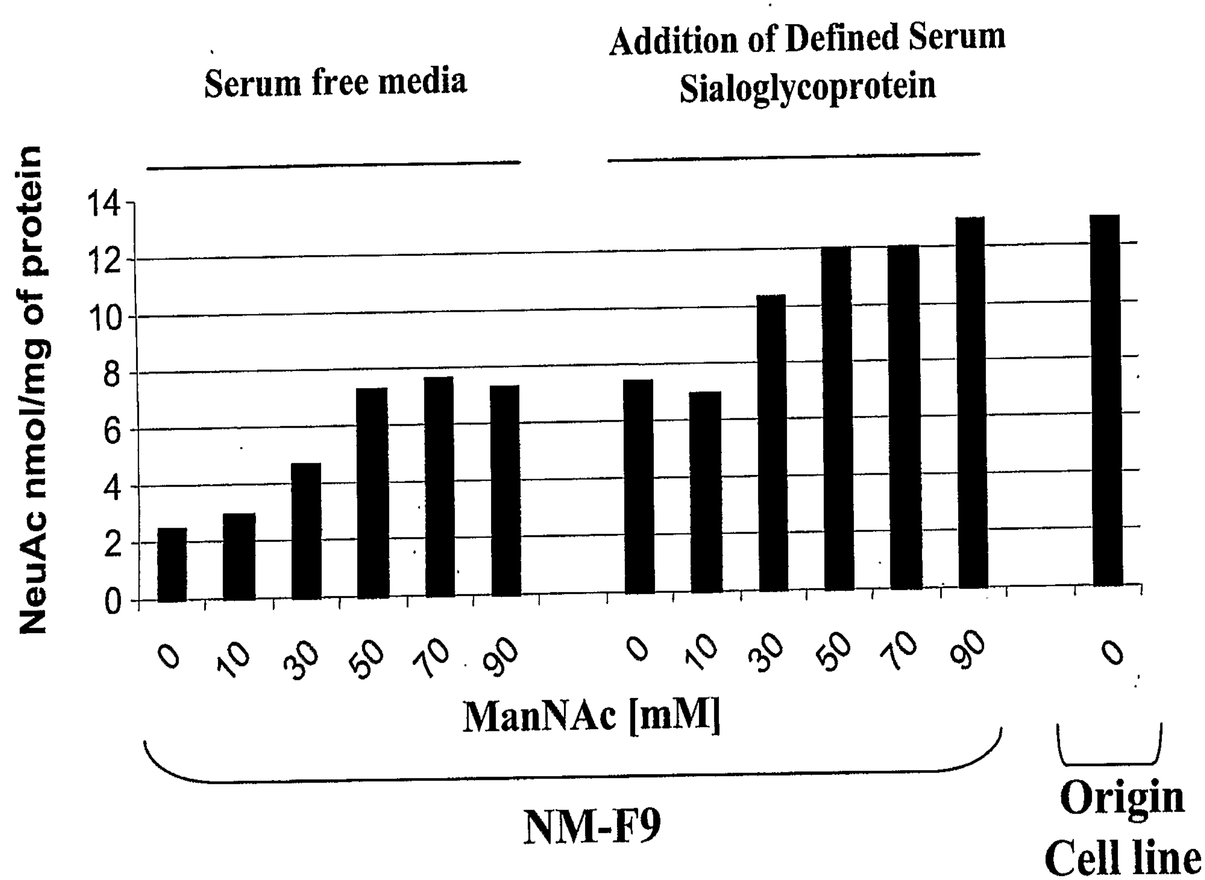

Metabolic Engineering using
Epimerase deficient human cell line
UDP-GlcNAc 2-Epimerase
UDP-N-Acetylglucosamine
N-Acetylmannosamine
ManNAc-6-P
NeuAc-9-P
NeuAc
CMP-NeuAc
Serum addition of
N-Acetylmannosamine
Sialyltransferases
Sialoglycoproteins
GLYCOTOPE

Fig. 24

Metabolic Engineering enables Gradual Sialylation

Thiobarbituric acid method: Determination of membrane glycoconjugate-bound sialic acid



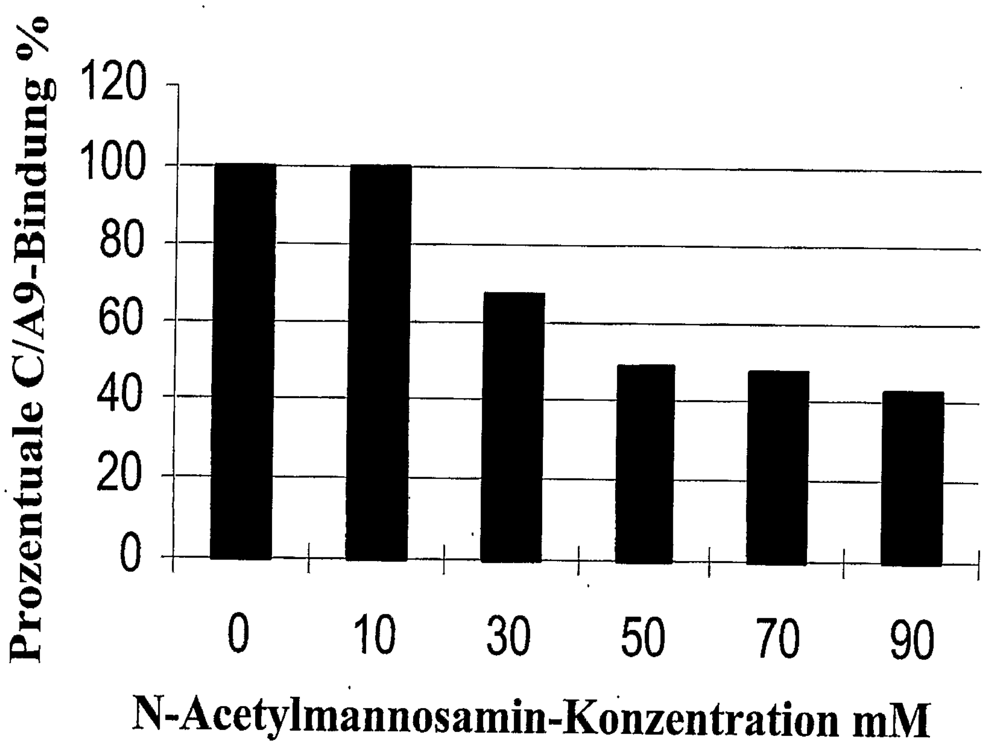
Cells were cultured in serum-free media supplemented with different concentrations of ManNAc + / - a defined serum sialoglycoprotein

Fig. 25

GLYCOTOPE

Durch metabolisches Engineering läßt sich der Sialinsäuregehalt auf dem Membranprotein

Different degrees of sialylation can be generated by defined amounts of sugar intermediates



Methode: Durchflußzytometrische Analyse mit einem Antikörper, der spezifisch für Sialinsäure-freies Asialoglykophorin A ist

Fig. 26

GLYCOTOPE

GlycoExpress

- Glycoengineered human cell line NM-F9 of myeloid leukemia origin
- Genetic defect in sugar nucleotide biosynthesis pathway
- Controlled regulation of sialylation by metabolic complementation
- Virus free
- Serum free
- Single cell cloning possible
- Biotechnologically favourable (suspension culture , 29 h doubling time)

Fig. 27

GLYCOTOPE

Summary (I)

GlycoProfiling using

mRNA expression analyses of glycosylation enzymes

analyses of enzyme activities

analyses of carbohydrate determinants

is a useful technique for selecting suitable cell lines for GlycoEngineering

- GlycoEngineering using

random mutanenesis in combination with

phenotypic selection

is a powerful technique for generating stable glycoengineered cell lines and cell products

Fig. 28

GLYCOTOPE

Summary (II)

TF-glycongineeed NM-F9 and NM-D4:

- genetic defect in the expression of UDP-GlcNAc 2-epimerase
- deficient in α2-3- and α2-6 sialylation
- sialylation can be reconstituted via intermediates of the sugar nucleotide biosynthesis pathway and sialoglycoproteins
- degree of sialylation can be controlled by defined sugar intermediates and sialoglycoproteins

$\Rightarrow$ basis for the development of a protein expression platform for the generation of human glycoproteins with fully human glycosylation and optimized sialylation

Fig. 29

GLYCOTOPE

Role of GM-CSF

Granulocyte Macrophage Colony Stimulating Factor

- Potent species-specific growth factor stimulating proliferation, development, differention and activation of granulocytes, macrophages, eosinophils and their progenitor cells
- Synergistic action with Epo in proliferation of erythroid and megakaryotic progenitor cells
- Enhances microbicidal activity, oxidative metabilism, phagocytotic activity and cytotoxicity of neutrophils, eosinophils and macrophages
- Induces release of histamins and leukotriene C4 from basophils
- Act synergistically with various other cytokines (e.g. IL-1, IL-3, IL-4, G-CSF)
- Differentiation and maturation of dendritic precursor cells which are central to humoral and cellular immune resposes

=> important stimulating factor for activation of innate and adaptive immune system

Fig. 30

GLYCOTOPE

Clinical Indications for human GM-CSF

Standard therapy:

- **Bone marrow transplantation**
  - reconstitution of hemotopoietic system
  - Expansion and collection of peripheral pool of stem cells
- **Neutropenia** after chemo- and/or radiotherapy
- **Cytopenia**, infections and hemorrhages after chemotherapy
- **AML** and **myelodysplastic syndromes** combinatorial anti-tumour GM-CSF/chemotherapy
- **Reconstitution of hemotopoietic system** (various diseases)

Experimental therapies:

- **Combined GM-CSF/radiotherapy** in cancer treatment abolishes lethal effects of irradiation and restores hematopoiesis
- **Combined GM-CSF/chemotherapy** for enhances tolerance to cytotoxic drugs (e.g. cancer chemotherapy, HIV)
- **Adjuvant** for cancer vaccines
- Cellular vaccines against cancer and infectious diseases
  - **Dendritic cell based vaccines** against tumours and infectious diseases
  - **Adoptive T cell transfer**
- Enhancement and **stimulation of innate immune system**

Fig. 31

GLYCOTOPE

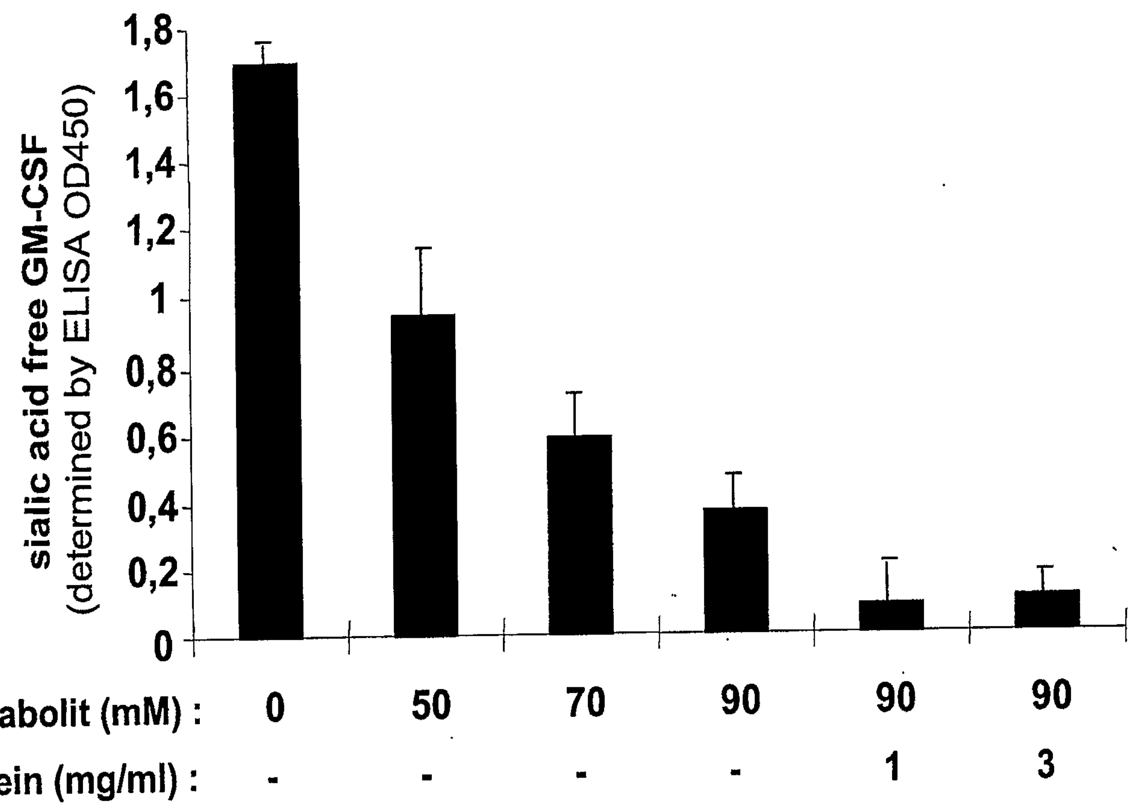
Recombinant human GM-CSF

- hGM-CSF is highly glycosylated by N- & O-glycans
- rhGM-CSF expressed in bacteria or yeast lacks any glycosylation or contains only glycans of a very different type (high mannose).
- Correct „human“ glycosylation & sialylation might affect hormone activity, half life during circulation and immunogenicity.
- State of the art: Glycosylation does not influence biological activity

Fig. 32

GLYCOTOPE

Differential sialylation of rhGM-CSF by metabolic engineering of NM-F9 cells

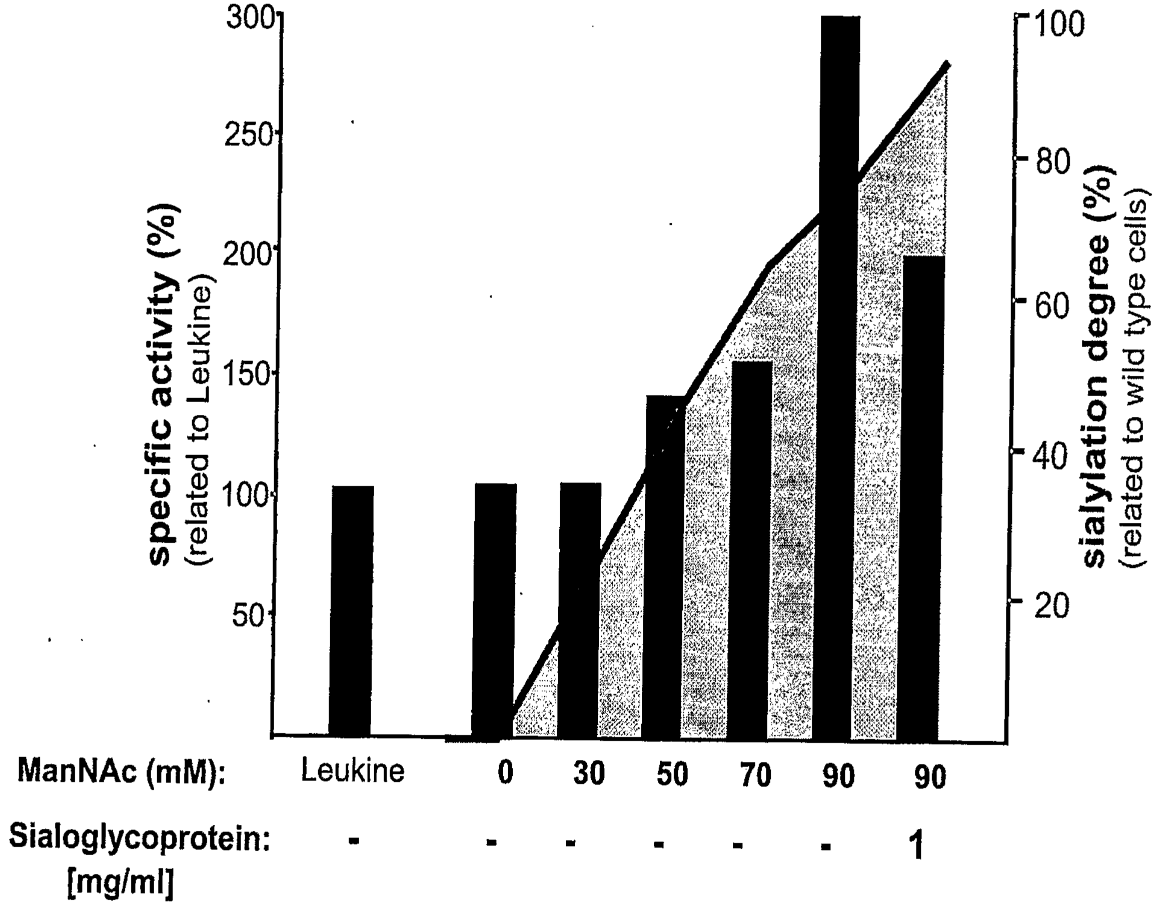


Sialic acid free GM-CSF was determined by ELISA using the anti-human GM-CSF monoclonal antibody for catching GM-CSF, 100 ng/ml NM-F9 cell culture derived GM-CSF and biotinylated peanut agglutinin (PNA) for detection of sialic acid free N- and O-glycans

Fig. 33

GLYCOTOPE

Differential sialylated rh-GM-CSF express different activities



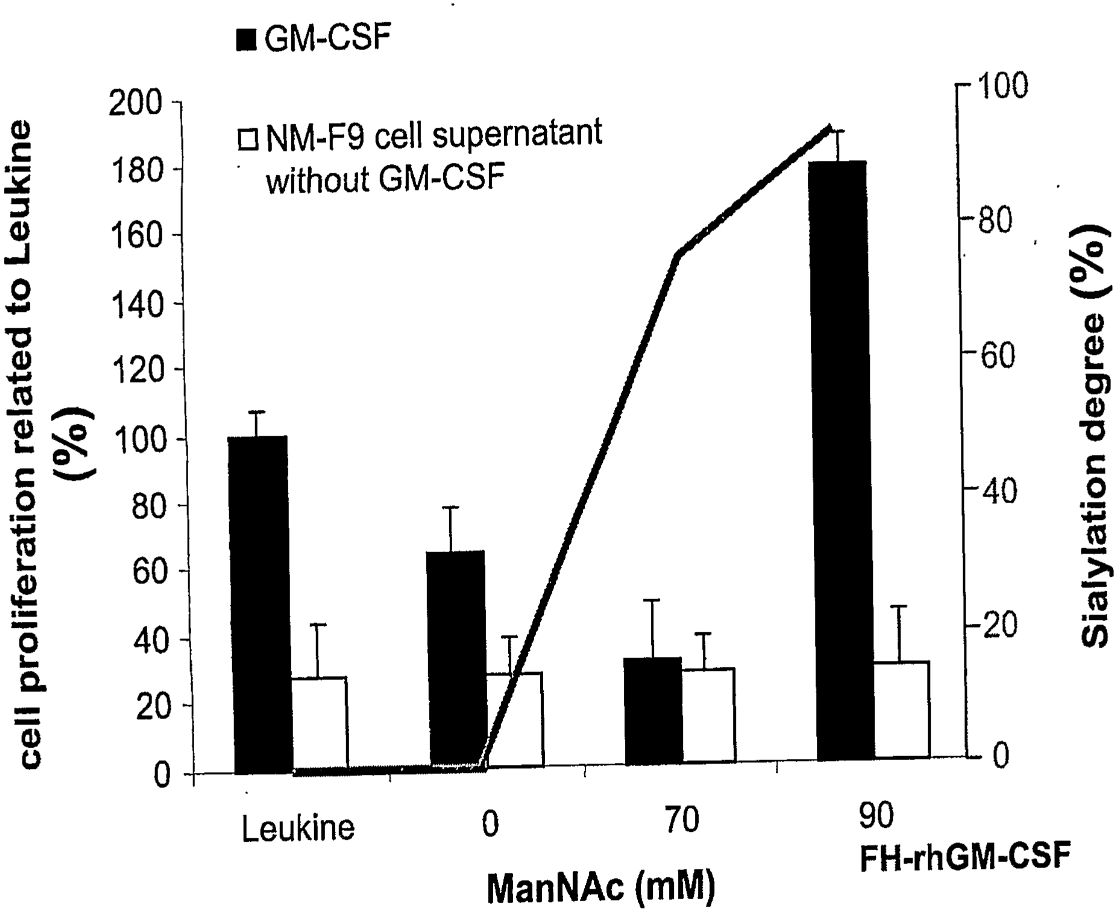
TF1 cells were cultured with NM-F9 supernatant of **5ng/ml** of **different sialylated rhGM-CSF** for 48 h. Cell proliferation was determined by BrdU-proliferation assay. NM-F9 supernatant without GM-CSF was used as control

rhGM-CSF (FH-GM-CSF) with a high but not the highest silylation degree is the most active growth factor in TF1 cells

Fig. 34

GLYCOTOPE

Highly sialylated fully human GM-CSF was most active for dendritic cells

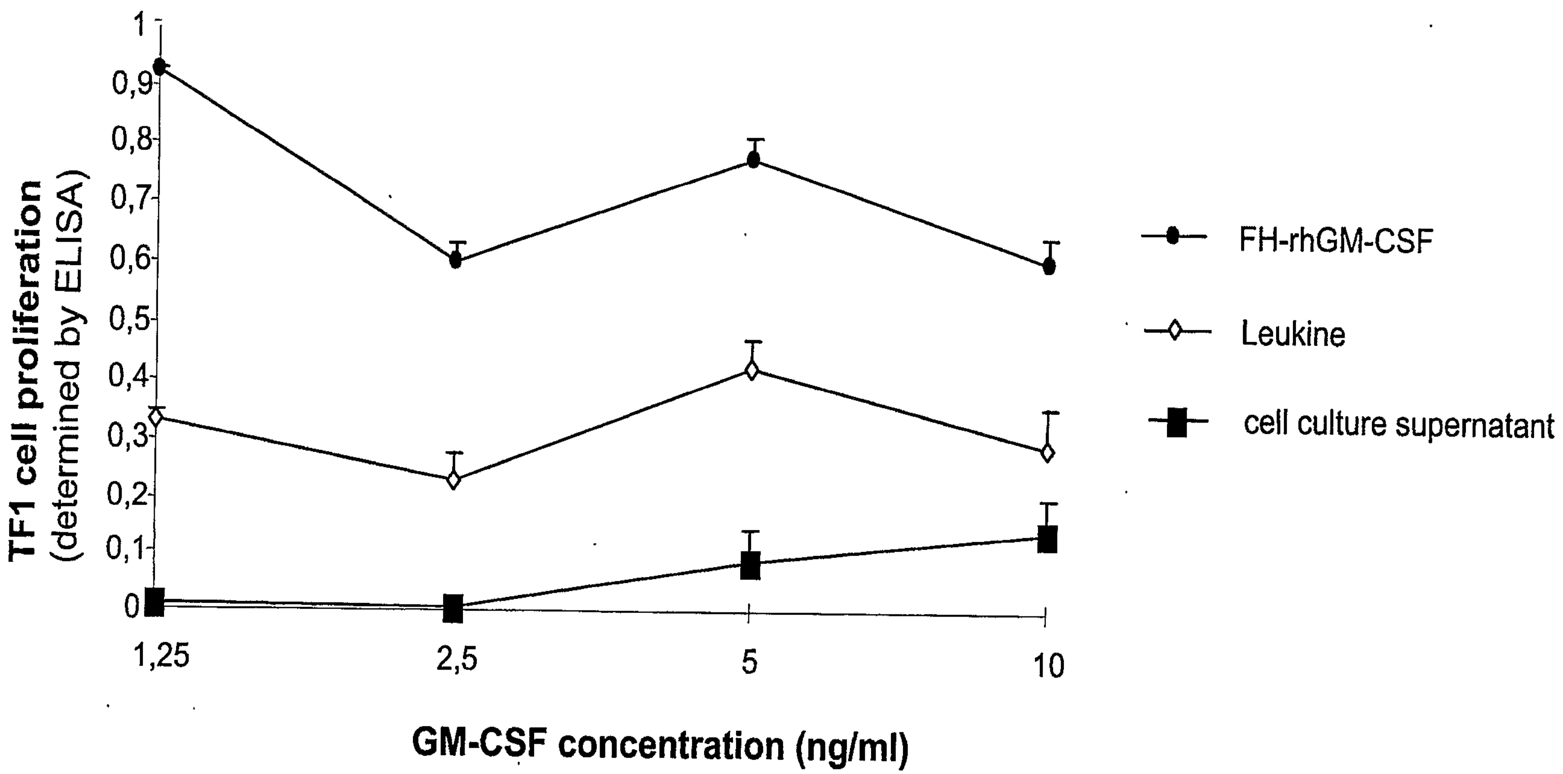


NemodDC cells were cultured with NM-F9 supernatants of **5ng/ml of different sialylated rhGM-CSF** for 24 h. Cell proliferation was determined by BrdU-proliferation assay.

Fig. 35 Different degrees of sialylation can have positive or negative effects on the activity of glycoproteins

GLYCOTOPE

Less fully human FH-GM-CSF is needed



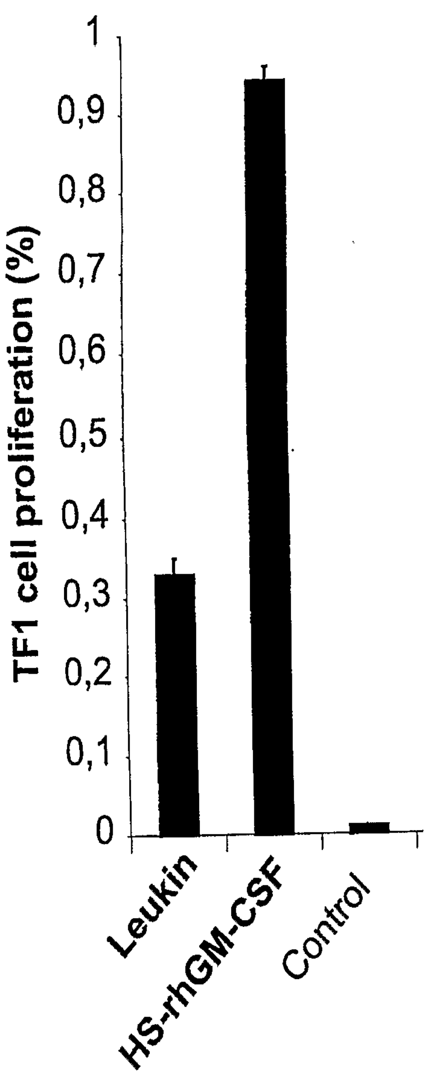
TF1-cells were cultured with **different concentrations of GM-CSF** for 48 h. Cell proliferation was determined by BrdU proliferation assay.

FH-rhGM-CSF (from NM-F9+90 mM sialic acid metabolite). Cell culture supernatant is without GM-CSF (from NM-F9+90 mM sialic acid metabolite)

Fig. 36

GLYCOTOPE

FH-rhGM-CSF is more active



- FH-GM-CSF is at least 3 times more active than commercial GM-CSF
- A longer serum half life and stability is expected
- No immunogenicity can be expected
- Lower doses and fewer administrations is expected
- Potentially less side effects
- Current clones produce
  1 - 1.5 µg FH-GM-CSF / ml 10^6 cells
  (Labscale; not optimized)

Fig. 37

GLYCOTOPE

Summary:

GlycoExpress is a potent expression technology for:

- expression of proteins with fully human glycosylation
- with optimized sialylation for improved bioactivity (activity, serum half-life and stability)
- the generation of new glycoproteins
- the generation new generations of improved biogenerics
- the investigating the role of human glycosylations and sialylations in the bioactivity

Fig. 38

GLYCOTOPE

Clinical Indications for human GM-CSF (detailled) (I)

Standard therapy:

- reconstitution of hemotopoietic system in patients
  undergoing autologous and allogenic bone marrow transplantation
  with delayed engraftment after bone marrow transplantation
  => shorter period of absolute neutropenia
  => fewer significant infections
  => diminished requirement for intravenous antibiotic administration
  => shorter inpatient hospitalization

- physiological reconstitution of hematopoiesis in all diseases characterized by aberrant maturation of blood cells or reduced production of leukocytes

- Treatment of life-threatening neutropenia following chemo and/or radiotherapy

- Treatment of cytopenia and cytopenia-related predisposition to infections and hemorrhages

- Expansion of peripheral pool of stem cells for collection (bone marrow transpl.)

- Induction of susceptibility of leukemia cells (AML and myelodysplastic syndromes) to cell-cycle specific drugs (chemotherapy)

Fig. 39

GLYCOTOPE

Clinical Indications for human GM-CSF (detailled) (II)

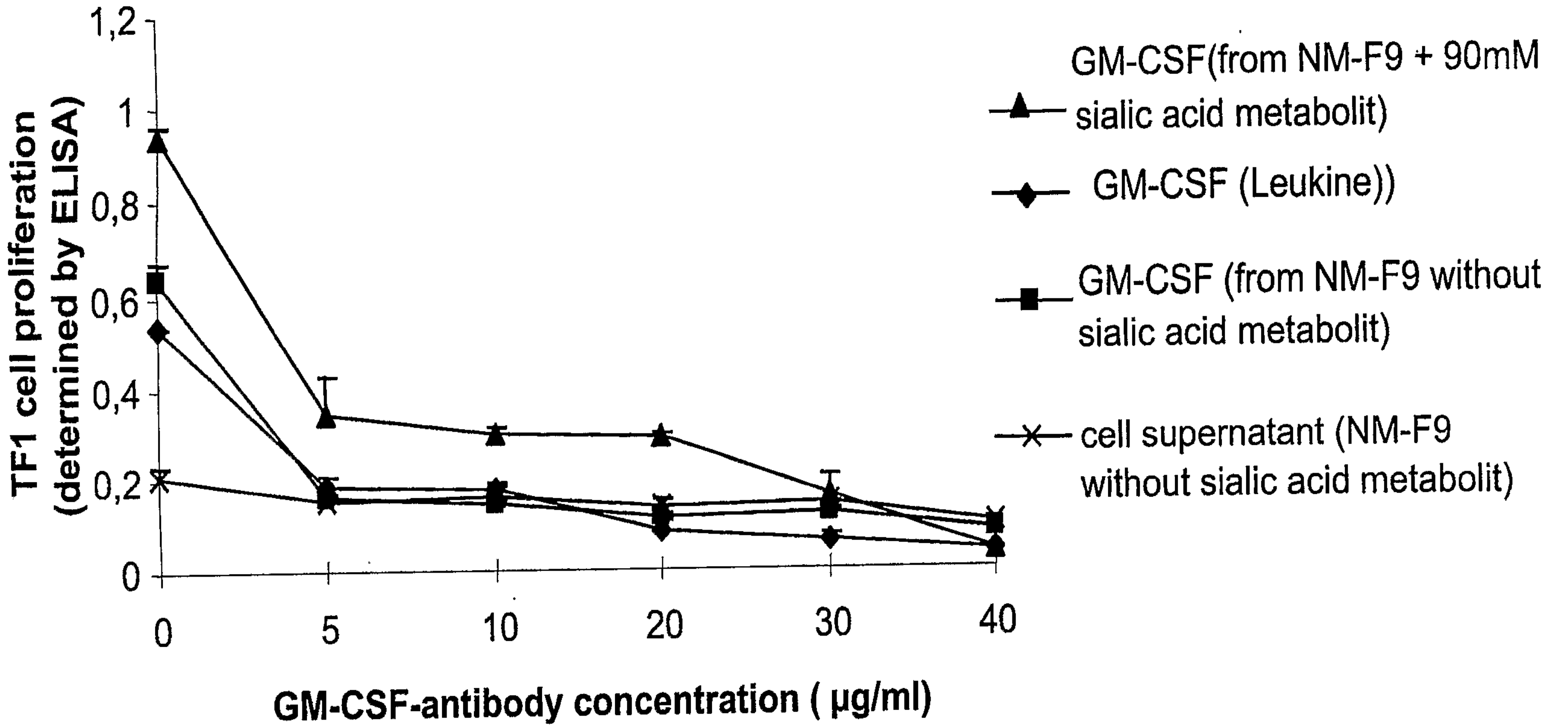
Experimental therapies:

- abolishment of effects of lethal irradiotion in radiotherapy and complete reconstitution of the hematopoiesis
- Enhances tolerance to cytotoxic drugs (e.g. cancer chemotherapy, HIV) enabling high or higher dosages and significant rediction of morbidity
- Adjuvant for cancer vaccines
- In combiantion with cellular vaccines against tumours and infectious diseases
  - Dendritic cell based vaccines
  - Adoptive T cell transfer
- Enhancement and stimulation of innate immune system
  (microbicidal activity, oxidative metabilism, phagocytotic activity, cytotoxicity of neutrophils and macrophages)

Fig. 40

GLYCOTOPE

HS-rhGM-CSF activity is specific and can be blocked by specific antibody



TF1-cells were cultured with supernatants of 5ng/ml GM-CSF for 48h and blocked with different concentrations of anti-human-GM-CSF-antibody BVD2-23 B6. Cell proliferation was determined by BrdU-proliferation assay.

Fig. 41

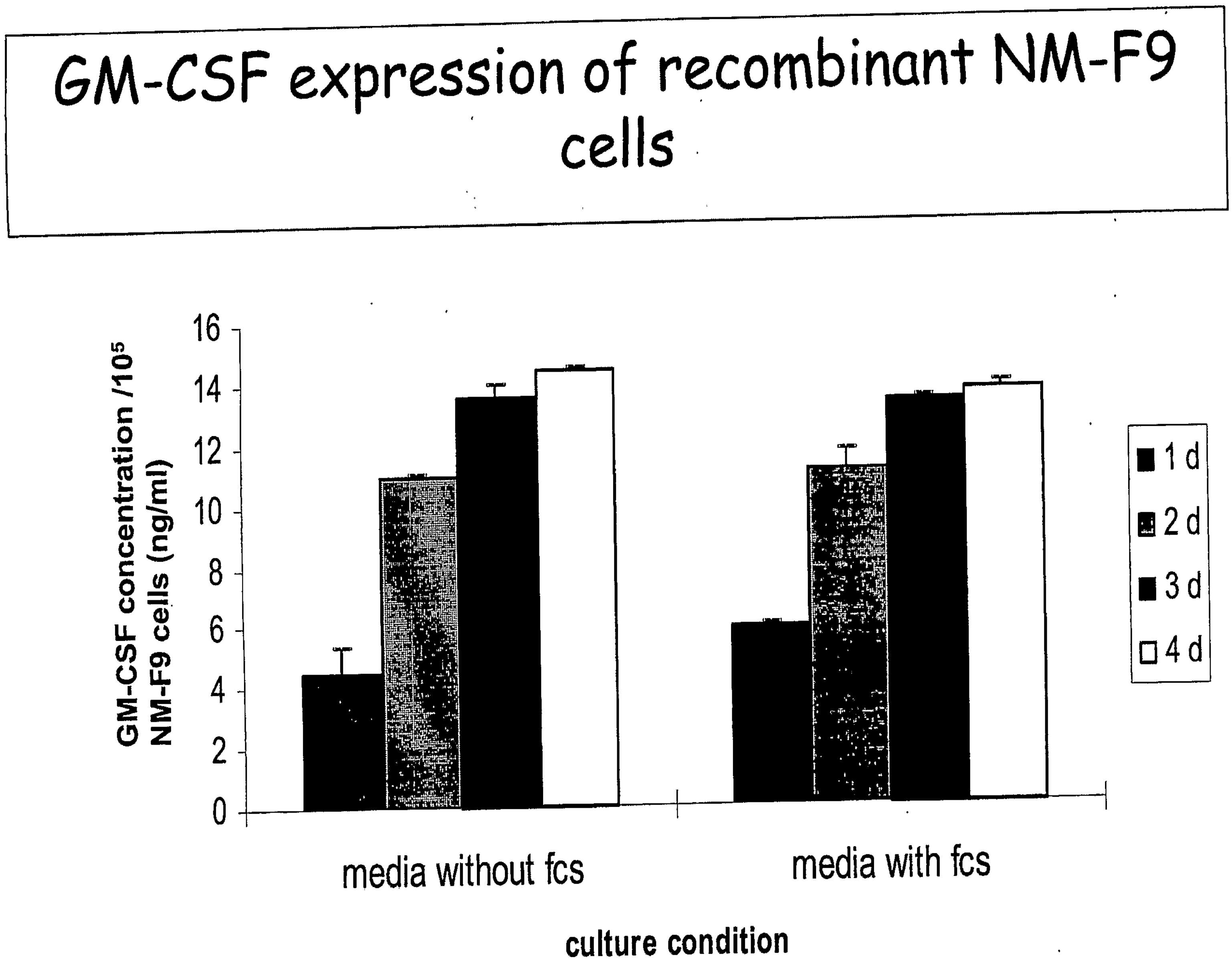
GLYCOTOPE

Strategy to increase the GM-CSF expression

- - generation of host NM-F9 cells with recombinase target sites located in regions of high transcription in genom
- - cloning of GM-CSF-gene in high expression vector with the same recombinase target site located in the genom of NM-F9 cells
- - recombinase-mediated integration of GM-CSF-gene in regions of high transcription in NM-F9 genom
- - 10- 20 times increase of GM-CSF expression
- (1-2µg/ml per 10^6 NM-F9 cells)

Fig. 42

GLYCOTOPE

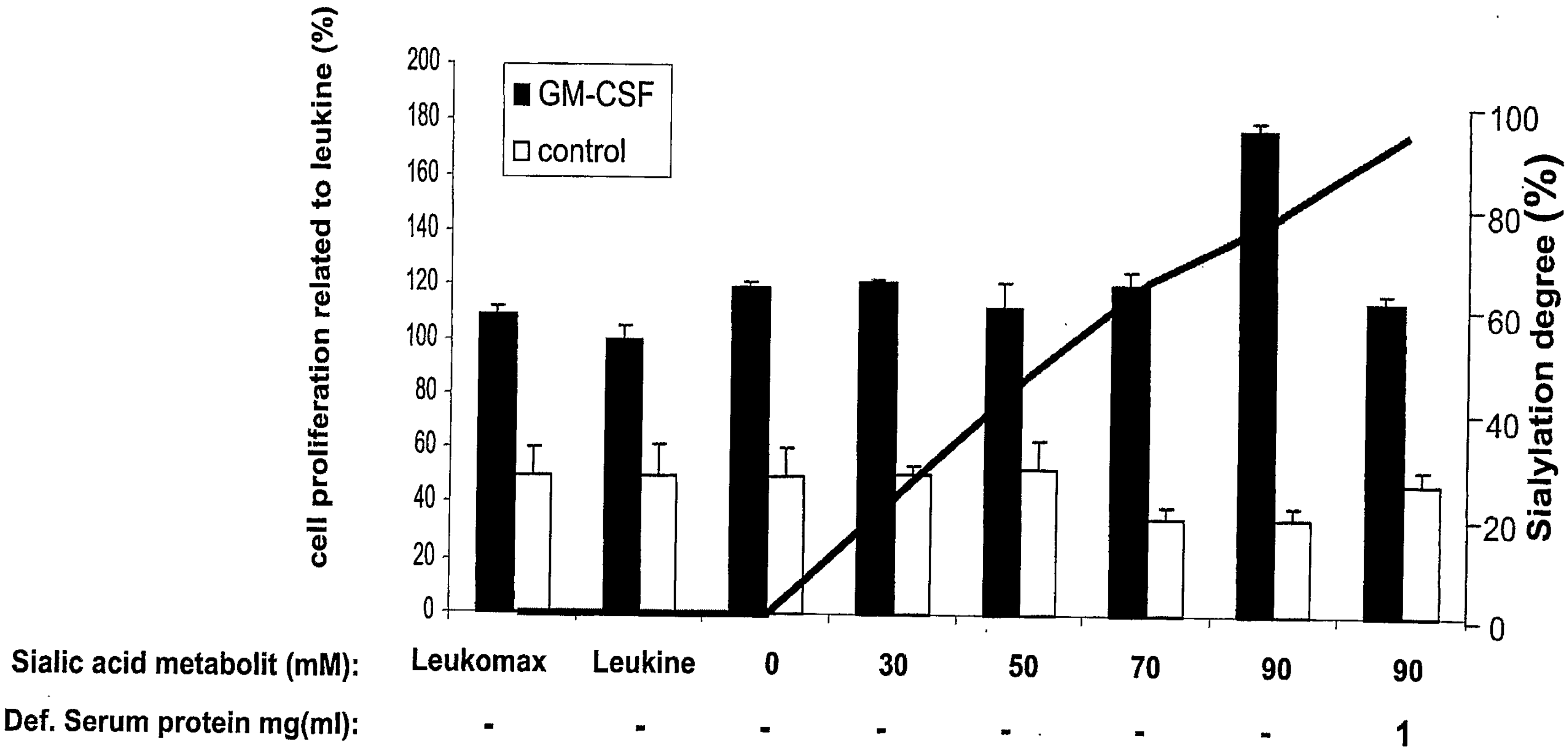


The GM-CSF concentration in the supernatant of recombinant NM-F9 cells were determined after 1d, 2d, 3d and 4d by ELISA. Cells were cultured in media supplemented with fcs and without fcs. After 4d 14ng GM-CSF/ 10^5 NM-F9 cells were secreted.

Fig. 43

GLYCOTOPE

Differential sialylated rh-GM-CSF express different activities



TF1 cells were cultured with NM-F9 supernatant of **5ng/ml** of **different sialylated rhGM-CSF** for 48 h. Cell proliferation was determined by BrdU-proliferation assay. NM-F9 supernatant without GM-CSF was used as control

Highly but not highest sialylated rhGM-CSF (FH-GM-CSF) is the most active growth factor in TF1 cells

GLYCOTOPE

Fig. 44